



JERTTA-RIINA SARKANEN

Engineering Vascularized Soft Tissue



ACADEMIC DISSERTATION

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ACADEMIC DISSERTATION

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Taaville

*In wilderness I sense the miracle of life
and behind it
our scientific accomplishments fade to trivia*

Charles Lindbergh (1902-1974)

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Abstract

Tissue engineering aims at replacing or regenerating tissue structures. In tissue engineering, the most limiting factor is the successful vascularization of the transplanted construct or the transplantation area. The current major hurdle is the lack of a therapeutic method that would rapidly induce adequate vascularization which would also remain in tissue. The proper method for induction of vascularization would help several tissue engineering applications as well as numerous patients suffering from ischemic diseases, chronic wounds and soft tissue defects. In addition to the tissue engineering applications, investigation of the angiogenesis process is important for the treatment of different other diseases such as cancers. Therefore, *in vitro* angiogenesis assays that can predict human effects are required. Understanding the role of angiogenesis in adipose tissue is of especial importance, as vasculature regulates both the adipose tissue mass development and adipose tissue reduction and obesity is a cause of a distruction in normal adipose tissue homeostasis.

The aim of the current study was to study angiogenesis and adipogenesis induction in *in vitro* assays and *in vivo*. In the first part of the study, acellular angiogenic and adipogenic agent was extracted from adipose tissue and the bioactivity of the extract was tested *in vitro* and *in vivo*. In cell culture studies, this adipose tissue extract was shown to stimulate angiogenesis and adipose tissue stromal cell maturation towards adipocytes. *In vivo*, when combined with hyaluronan hydrogel, the extract was shown to induce sustained soft tissue formation. No hypersensitivity or foreign body reactions were seen. Adipose tissue extract has therefore potential to be used as an acellular alternative in the treatment of soft tissue defects for reintroducing soft tissue at the defect sites. Adipose tissue extract has also potential to be used to induce revascularization in ischemic tissues and to be used in other tissue engineering products that fail due to inadequate vascularization. Moreover, adipose tissue extract can be used as an inducer in an *in vitro* model of natural adipogenesis.

The second part of the study focused on developing *in vitro* methods for angiogenesis induction. We created a multilayered adipose stromal cell vascular network with properties of maturing vessels that can be used for studying angiogenesis *in vitro*, and especially, in the development of *in vitro* three dimensional tissue models as well as possibly as a vascularized platform in implantable soft tissue constructs. We also intra-laboratory validated an *in vitro* angiogenesis assay to be used as a routine cell assay for drug and chemical screening. The currently validated *in vitro* assay is a

relevant-to-human bioassay that can be used for preclinical drug efficacy screening studies, and in addition, is applicable also for testing angiotoxicity of chemicals.

Tiivistelmä

Kudosteknologia pyrkii korvaamaan tai tuottamaan uusia kudusrakenteita. Suurin ongelma kudosteknologisten sovellusten kehittämisessä on verisuonituksen puuttuminen ja riittävän verisuonituksen muodostuminen. Sellainen terapeutinen menetelmä, joka aikaansaisi nopean verisuonien muodostumisen korjattavalla alueella ja jonka aikaansaama uudisverisuonitus pysyisi kudoksessa pitkään, hyödyttäisi lukuista joukkoa niin pehmytkudosvammoista kärsiviä potilaita kuin kroonisten haavojen tai iskeemisten kudosten hoitoa tarvitsevia potilaita. Verisuonituksen muodostumisen ja sen estämisen tutkiminen on lisäksi tärkeää useissa muissa sairauksissa kuten syövässä, ja tämä vuoksi ihmiskudoksen verisuonitusta mallittavia solutason testimenetelmiä verisuonimuodostuksen tutkimiseksi tarvitaan. Verisuonimuodostuksen tutkiminen rasvakudoksessa on erityisen tärkeää siksi, että verisuonitus säätelee rasvakudoksen määrää ja lihvuuteen johtavaa rasvakudoksen liiallista kertymistä.

Tässä tutkimuksessa tutkittiin verisuonituksen ja pehmytkudoksen syntymistä. Rasvakudoksesta eristettiin solutonta verisuonituksen ja rasvan muodostumista edistävää materiaalia, rasvakudosekstraktia, ja tutkittiin sen kykyä indusoida sekä verisuonitusta että pehmytkudoksen muodostumista soluviljelmissä ja implantointikokein. Soluviljelmissä rasvakudosekstraktin todettiin aikaansaavan verisuonirakenteiden muodostumista sekä triglyseridien kertymistä rasvakudoksen kantasoluihin. Implantointikokeissa materiaalia yhdistettiin hyaluronihappohydrogeeliin ja todettiin, että rasvakudosekstrakti indusoi nopeaa verisuonituksen muodostumista ihonalaiskudoksessa, sekä kypsän, pysyvän rasvakudoksen syntymistä. Materiaalin todettiin olevan kudosityhteensopiva. Täten työssä tutkittu rasvakudosekstrakti on lupaava soluton hoitomuoto verisuonimuodostuksen ja rasvakudoksen aikaansaamiseksi pehmytkudosvaurioissa. Rasvakudosekstraktia voidaan käyttää lisäksi verisuonituksen parantamiseen erilaisissa kudosteknologisissa sovelluksissa. Rasvakudosekstraktia avulla voidaan mallintaa luonnollisen rasvamuodostuksen mallittamiseen soluviljelmässä.

Työn toisessa osassa tutkittiin ja kehitettiin ihmissolumalleja verisuonituksen muodostumisen tutkimiseen. Tutkimuksessa kehitettiin rasvakudoksen kantasoluihin perustuva kolmiulotteinen verisuonimalli, jota voidaan käyttää verisuonitutkimuksessa tai luotaessa edistyneitä kudosmalleja soluviljelmissä tai tehtäessä kudusrakenteita

soluterapiatarkoituksiin. Työssä kehitettiin lisäksi laadultaan korkeatasoinen ja toistettava testimenetelmä käytettäväksi prekliinisen vaiheessa verisuonimuodostuksen tutkimiseksi lääkeaineiden ja kemikaalien turvallisuuden ja tehon testausta varten.

Abbreviations

AB/AM	antibiotic antimycotic mixture
ACBP	acyl-coenzyme A – binding protein
Ang-1	angiopoietin 1
Ang-2	angiopoietin 2
ANOVA	analysis of variance
AgRP	agouti-related protein
ap2	fatty acid binding protein
APC	allophycocyanin
ASC	adipose stromal cell
ATE	adipose tissue extract
Axl	AXL receptor tyrosine kinase
BMI	body mass index
BMP-2	bone morphogenetic protein 2
BMP-4	bone morphogenetic protein 4
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCL5 (RANTES)	chemokine (C-C motif) ligand 5
C/EBP α	CCAAT/enhancer binding protein α
C/EBP β	CCAAT/enhancer binding protein β
C/EBP δ	CCAAT/enhancer binding protein δ
CNTF	ciliary neurotrophic factor
CREB	cyclic adenosine monophosphate responsive element binding protein
COL I	collagen I
COL III	collagen III
COL IV	collagen IV
COL XVIII	collagen XVIII
CTACK	cutaneous T cell-attracting chemokine
CV	coefficient of variation
DLL4	delta-like ligand 4
Dtk	growth factor receptor tyrosine kinase
DMEM	Dulbecco's Modified Eagle's Medium
DMEM/F12	Dulbecco's modified Eagle's medium: Nutrient mixture F-12
DMSO	dimethyl sulfoxide
EBM-2	endothelial cell basal medium -2
ECM	extracellular matrix
EGF	epidermal growth factor

EGM-2	endothelial cell growth medium -2
EGF-R	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ENA-78	epithelial neutrophil activating peptide-78
FAS	fatty acid synthase
Fas/TNFRSF6	Fibroblast-associated/Tumour necrosis factor receptor superfamily member 6
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FGF	fibroblast growth factor
Flk-1	Fetal liver kinase-1, transmembrane tyrosine kinase, vascular endothelial growth factor receptor 2
Flt-1	Fms-like tyrosine kinase 1, vascular endothelial growth factor receptor 1
Flt-3 ligand	Fms-like tyrosine kinase 3 ligand
bFGF	basic fibroblast growth factor
FGF-4	fibroblast growth factor 4
FGF-6	fibroblast growth factor 6
FGF-9	fibroblast growth factor 2
GFP	green fluorescence protein
GLP	good laboratory practice
GLUT4	glucose transporter 4
HA	hyaluronic acid
hASC	human adipose stromal cells
hATE	human adipose tissue extract
HGF	hepatocyte growth factor
hpf	high power field
hr	hour
hrs	hours
HS	human serum
HUVEC	human umbilical vein endothelial cells
IFN- γ	interferon γ
IGF-I	insulin-like growth factor I
GCSF	granulocyte colony-stimulating factor
GH	growth hormone
GITR	glucocorticoid-induced tumor necrosis factor receptor
GITR-ligand	glucocorticoid-induced tumor necrosis factor receptor ligand
GRO	cytokine-induced neutrophil chemoattractant 1 growth related oncogene
HE	hematoxylin eosin
IGF-1	insulin-like growth factor 1
IGF-1 SR	insulin-like growth factor 1 soluble receptor
IGFBP-3	insulin-like growth factor binding protein 3

IGFBP-6	insulin-like growth factor binding protein 6
IL-1 β	interleukin 1 β
IL-6	interleukin 6
IL-8	interleukin 8
IL-10	interleukin 10
IL-11	interleukin 11
iNOS	inducible nitric oxide synthase
ISO	International Organization of Standardization
I-TAC	Interferon-inducible T-cell alpha chemoattractant
L-glut	L-glutamine
LIF	leukemia inducible factor
LIGHT	homologous to lymphotoxin, exhibits inducible expression, and competes with HSV glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes
MCP-1	monocyte chemoattractant protein-1
MCP-2	monocyte chemoattractant protein-2
MCP-3	monocyte chemoattractant protein-3
MEM	minimal essential medium
MIF	macrophage migration inhibitory factor
min	minutes
MIP-1 α	macrophage inflammatory protein-1 α
MIP-1 β	macrophage inflammatory protein-1 β
MIP-3 β	macrophage inflammatory protein-3 β
MSP- α	macrophage stimulating protein α
NEAA	non essential amino acids
NAP-2	neutrophil activating protein-2
NF-68	neurofilament 68
NGF	nerve growth factor
NR	neutral red
NRU	neutral red uptake
NT-4	neurotrophin 4
OECD	Organisation for Economic Co-operation and Development
ORO	oil-red-O
PAI-1	plasmin activator inhibitor 1
PBS	phosphate buffered saline
PDGF-B	platelet derived growth factor beta
PDGFR β	platelet derived growth factor receptor beta
PE	phycoerythrin
PECAM-1	platelet/endothelial cell adhesion molecule-1
PIGF	placental growth factor
P/S	penicillin/streptomycin
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
rATE	rat adipose tissue extract

RANTES	regulated upon activation, normal T-cell expressed and secreted)/CCL5 (CC chemokine ligand 5
RNA	ribonucleic acid
RPLP0	ribosomal protein large P0
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
α SMA	alfa smooth muscle actin
SMMHC	smooth muscle myosin heavy chain
SREBP1c	sterol-regulatory element binding protein 1c
SVF	stromal vascular fraction
TGF- β	transforming growth factor- β
Tie-1	tyrosine kinase (T) with Ig (I) and epidermal (E) growth factor homology domain 2
Tie-2	tyrosine kinase (T) with Ig (I) and epidermal (E) growth factor homology domain 2
TIMP-1	tissue inhibitor of matrix metalloproteinase-1
TIMP-2	tissue inhibitor of matrix metalloproteinase-2
TNF- α	tumor necrose factor α
tPA	tissue-type plasminogen activator
TRAIL R3	tumor necrosis factor-related apoptosis-inducing ligand receptor 3
TRAIL R4	tumor necrosis factor-related apoptosis-inducing ligand receptor 4
TRITC	tetramethyl rhodamine isothiocyanate
Tsp-1	thrombospondin 1
Tsp-2	thrombospondin 2
UBS	umbilical cord buffer solution
uPA	urokinase-type plasminogen activator
VEGF(-A)	vascular endothelial growth factor
VEGF-B	vascular endothelial growth factor B
VEGF-C	vascular endothelial growth factor C
VEGF-D	vascular endothelial growth factor D
VEGFR-1	vascular endothelial growth factor receptor 1
VEGFR-2	vascular endothelial growth factor receptor 2
VEGFR-3	vascular endothelial growth factor receptor 3
vWf	von Willebrand factor

Abbreviations are defined at first mention in the review of the literature (and used only for concepts that occur more than once).

List of original publications

The present study is based on the following original publications, referred to in the text by their Roman numerals (I-IV):

- I) Sarkanen JR, Kaila V, Mannerström B, Rätty S, Kuokkanen H, Miettinen S and Ylikomi T (2012) Human adipose tissue extract induces adipogenesis and angiogenesis *in vitro*. Tissue Eng Part A. 18:17-25. Epub 2011 Oct 4.
- II) Sarkanen JR, Ruusuvuori P, Kuokkanen H, Paavonen T and Ylikomi T (2012) Bioactive acellular implant induces angiogenesis and adipogenesis and sustained soft tissue restoration *in vivo*. Tissue Eng Part A. Jun 28. Epub ahead of print.
- III) Sarkanen JR, Vuorenpää H, Huttala O, Mannerström B, Kuokkanen H, Miettinen S, Heinonen T and Ylikomi T (2012) Adipose Stromal Cell Angiogenesis *in vitro* Model Provides a Versatile Tool for Tissue Engineering and Vascular Research. Cell Tissues Organs. Jun 27. Epub ahead of print.
- IV) Sarkanen JR, Mannerström M, Vuorenpää H, Uotila J, Ylikomi T and Heinonen T (2010) Intra-laboratory prevalidation of a human cell based *in vitro* angiogenesis assay for testing angiogenesis modulators. Front Pharmacol 1:147. Epub 2011 Jan 20.

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Introduction

There is a tremendous need for tissue engineered organs. The loss of tissue or failure of organ is currently one of the most common and most expensive challenges to clinical health care (Laschke et al. 2006). The demand for organ and tissue transplants is much higher than the availability, and every year thousands of patients die while waiting for a suitable organ (Jain et al. 2005). The field of tissue engineering has emerged in the 1980s with the aim of creating biological substitutes for restoring human tissues. The tissue engineering could provide help for a number of diseases, but several challenges exist, such as isolation and expansion of specific cells, the organization of cells in constructs, as well as the optimal microenvironment for growth and differentiation (Jain et al. 2005).

The most crucial problem in tissue engineering is the vascularization of the tissue constructs (Jain et al. 2005; Wu et al. 2007; Rivron et al. 2008), and inadequate vascularization limits the size of engineered structures (Moiola et al. 2010; Patel and Mikos 2004; Wu et al. 2007). Until recently, the tissue engineered structures have been avascular tissues like skin (Kremer et al. 2000) and cartilage (Vacanti and Upton 1994). An effective method for angiogenesis induction is urgently needed, and the ability to vascularize tissue constructs would be a crucial step in tissue engineering (Jain et al. 2005). Techniques for vascularization can be divided into two: *in vitro* and *in vivo* engineered vascular networks (Lokmic and Mitchell 2008). In both situations appropriate cells or their precursors, extracellular matrix and proangiogenic microenvironment, are needed for the development of vascular structures (Lokmic and Mitchell 2008).

The stimulation of blood vessel formation *in vivo* has been tried to be improved by biomaterial modifications and by addition of glycosaminoglycans or growth factors into bioscaffolds (Nillesen et al. 2007). The use of vascular endothelial growth factor (VEGF) has been promising, however, not sufficient alone to create mature and stable vasculature (Blau and Banfi 2001). The use of a cocktail of growth factors e.g. VEGF, placental growth factor (PIGF), Angiopoietin-1 (Ang-1), platelet derived growth factor beta (PDGF-B) and transforming growth factor- β (TGF- β), has been more successful in therapeutic angiogenesis (Jain et al. 2005). Nevertheless, finding the optimal cocktail and adequate factors for inducing angiogenesis in tissue remains an unsolved task (Jain et al. 2005). Another solution is to insert the cellular components of vessels, i.e. endothelial cells and pericytes directly to the tissue graft. These cells

assemble into vascular structures *in vivo* and/or induce the host vasculature remodeling and neovascularization by secreting angiogenic growth factors (Jain et al. 2005). A problem with cell-seeded grafts is the cell death associated with implantation (Koc and Gerson 2003). However, *in vitro* prevascularization of certain tissue constructs has shown to improve their functionality *in vivo* (Levenberg et al. 2005).

Adipose tissue engineering has traditionally focused on restoring the volume loss with no or much less emphasis on tissue function (Vermette et al. 2007). Tissue engineered fat *in vitro* and *in vivo* has been studied tremendously during the last decade, but most current therapy approaches for soft tissue induction, due to inadequate vascularization (Nillesen et al. 2007; Verseijden et al. 2009; Wu et al. 2007), fail to produce satisfactory, reproducible and sustained result (Patrick 2001).

Angiogenesis is suggested to be the “organizing principle in biology and medicine” (Folkman 2007). Already at 1970s Folkman presented findings that in the absence of vascularization tumors could not obtain larger diameter than 2-3 mm (Folkman 1971) and that the growth of tissue was dependent on neovascularization which was mediated by stimulating factors (Folkman 1971; Folkman 1990). In addition to tissue engineering applications, the investigation of induction of vascularization is important in numerous other normal and pathological conditions. To succeed in angiogenesis induction, we must be able to create and mimic the biochemical cocktail of blood and the biophysical environment during blood flow (Ko et al. 2007). In this study, the development of vascularization *in vitro* and *in vivo* as well as inductive adipogenesis, are of especial interest.

Review of the literature

Vascular network

Vascular network regulates the body homeostasis by transporting oxygen, liquids, nutrients, cells and signaling molecules to all parts of the body and by disposing waste from tissues (Rivron et al. 2008). The circulatory system aids immune system cells to protect the body from harmful attacks and contributes to the control of body temperature and blood pressure (Ko et al. 2007). The vascular system also aids in tissue regeneration and in the communication between organs (Rivron et al. 2008). As blood vessels are spread throughout the body, the changes in normal vessel growth and maintenance contribute to a wide number of diseases (Carmeliet and Jain 2011).

The cardiovascular system is the first system to develop in the embryo (Haigh 2008). The adult vasculature has a total surface area of approximately 1000 m² (Buschmann and Schaper 1999). The smallest blood vessels are capillaries, consisting of a lumen with an inner diameter of 4-10 µm, and a surrounding wall of endothelial cells (Ko et al. 2007). Capillaries are the main site of gas and nutrient exchange (Rivron et al. 2008). The difference between capillaries and arterioles is the appearance of smooth muscle cells in arterioles. Arterioles have a diameter of 10-300µm, and they contain 1-2 layers of smooth muscle cells in addition to the endothelial cell layer. Post-capillary venules lack smooth muscle cells, but differ from capillaries with the inner diameter of a lumen, that is 10-50 µm for the post-capillary venules. Larger venules are called collecting venules, with an inner diameter of 50-300 µm. (Ko et al. 2007)

Blood vessel formation

Blood vessels are formed of endothelial cells, extracellular matrix (ECM) and mural cells. Endothelial cells form a permeable tubule layer connected with tight junctions. Tubules are surrounded by mural cells, i.e. either pericytes in capillaries, or pericytes and smooth muscle cells in arterioles and arteries. Pericytes stabilize endothelial cells by releasing factors such as VEGF and Ang-1. (Rivron et al. 2008) The adult cells also contain oxygen sensors prolylhydroxylase and hypoxia-inducible factors (HIF-2α). They

allow the vessels to adjust to changes in human body and to optimize the blood flow. (Carmeliet and Jain 2011)

Normal blood vessel formation can occur by three different processes: vasculogenesis, angiogenesis and arteriogenesis. Vasculogenesis appears in the embryonic development, when mesodermal cells differentiate into angioblasts or hemangioblasts. These endothelial precursor cells differentiate into endothelial cells which then organize into primary capillary plexuses and further into vascular networks. (Moon and West 2008; Papetti and Herman 2002; Risau 1997) Angiogenesis is the formation of new capillary sprouts from the pre-existing blood vessels (Carmeliet and Jain 2011; Risau 1997). Angiogenesis is mainly driven by tissue hypoxia signals and occurs under conditions that belong to normal life cycle and retain normal body homeostasis such as menstrual cycle (folliculogenesis and ovulation), pregnancy, fracture repair and wound healing. Angiogenesis also occurs in pathological disease conditions such as in tumor growth and metastasis, in rheumatoid arthritis, in ischemic diseases, in neonatal hemangiomas, in hypertrophic scars or keloids, in atherosclerosis and in retinopathies (Beer et al. 1998; Buschmann and Schaper 1999; Carmeliet and Jain 2011; McHoney 2010). Arteriogenesis occurs after occlusion of a major artery, and is defined as a rapid proliferation of pre-existing arteries. It allows the pre-existing arteries to bypass the occlusion site. (Buschmann and Schaper 1999) Arteriogenesis and angiogenesis share many properties such as growth factor induction, but arteriogenesis is dependent on inflammation, not on hypoxia, as angiogenesis is. (Buschmann and Schaper 1999)

Tumors can use also additional processes for abnormal blood vessel formation, such as vascular mimicry, where tumor vessels form abnormal vascular-like structures themselves, or vessel co-option, where tumor blood vessels incorporate into normal tissue capillaries. Tumor stem cells themselves have also ability to differentiate into abnormal endothelial cells. (Carmeliet and Jain 2011)

This review will focus on physiological angiogenesis and its regulation, and on angiogenesis in adipose tissue.

Angiogenesis

In adults, new blood vessels arise mainly by sprouting from the existing vasculature (Carmeliet 2005). In sprouting angiogenesis, proliferative endothelial cells retain their basal-luminal polarity (Paku et al. 2011) Angiogenesis can also occur by intussusceptive growth of existing vessels (Carmeliet 2005; Rivron et al. 2008). Intussusceptive angiogenesis is rapid and is mainly characterized by the insertion of endothelial cell bridges and connective tissue columns across the capillary lumen (Paku et al. 2011). The pre-existing vessels therefore partition into daughter vessels which results

in an increase in the capillary density (Carmeliet and Jain 2011; Paku et al. 2011).

Endothelial cell migration

After receiving angiogenic signal such as VEGF, VEGF-C, Ang-2 or FGFs from a hypoxic, inflammatory or tumor cell, pericytes detach from the vessel wall. The basement membrane and ECM are degraded by matrix metalloproteases (MMPs) and by suppression of protease inhibitors (tissue inhibitor of matrix metalloproteases, TIMPs). VEGF increases the permeability of the endothelial cell layer and loosens the endothelial tight junctions. The vessel is then dilated by nitric oxide synthase. (Carmeliet and Jain 2011) Plasma proteins are leaked from loosened vessels and they provide a provisional ECM to the developing vessels (Jain 2003). Endothelial cells lose contact with basement membrane laminin and become exposed to collagen I, which activates cytoskeleton reorganization and endothelial sprouting (Davis and Senger 2005; Rhodes and Simons 2007). In a healthy adult, quiescent endothelial cells have half-lives of thousands of days (Carmeliet and Jain 2011; Fan et al. 1995). However, in angiogenesis, activated endothelial cells have a half-life of only a few days (Fan et al. 1995). Endothelial cells become motile and align into chords. One endothelial cell is differentiated to be the leading cell, a tip cell. The tip cell starts to migrate towards the VEGF stimulation signal from extracellular matrix. The VEGF gradient induces tip cell's neighbors, the stalk cells, to proliferate and elongate the vessel sprout and to form the lumen. (Carmeliet and Jain 2011; Gerhardt et al. 2003; Ruhrberg et al. 2002) VEGF induces expression of delta-like ligand (DLL-4) in tip cells. DLL-4 binds to its receptors Notch 1 and Notch 4 in stalk cells. (Carmeliet and Jain 2011) This contact downregulates VEGFR-2 expression in stalk cells and they become less responsive to VEGF. This ensures that only one cell, the tip cell, leads the tubule formation, that vessel is orderly developed and the excess angiogenesis is prevented. (Carmeliet and Jain 2011; Chung et al. 2010) The tip cell expresses membrane type 1 matrix metalloprotease (MT1-MMP) that opens up the matrix for the endothelial sprout. MT1-MMP is later down-regulated when stalk cells come into contact with pericytes. (Chung et al. 2010) Endothelial cells are sealed with tight cell-cell junctions and adherens junctions. VE-cadherin is an important component of endothelial cell-to-cell junctions, neural (N) -cadherin facilitates endothelial cell-mural cell communication and gap junctions exist between endothelial cells and endothelial cells and pericytes. (Jain 2003)

Pericytes and maturation of the vessels

Vessels are stabilized by recruiting mural cells to the vessel wall and generating ECM. Mature capillaries are partially covered by mural cells called pericytes and larger vessels are covered by vascular smooth muscle cells and pericytes (Gerhardt and Betsholtz 2003). Pericytes are cells that are located in between the endothelium and the surrounding tissue and make contacts with the endothelium (Armulik et al. 2005). Pericytes are important regulators of vascular maturation, stabilization and remodeling (Armulik et al. 2005). Pericytes are morphologically diverse cells depending on the tissue and the stage of development. Some of the markers that pericytes express are smooth muscle α -actin (α -SMA), desmin, platelet derived growth factor receptor- β (PDGFR- β), proteoglycan NG-2, aminopeptidases A and N, RGS5 and *XlacZ4* gene (Gerhardt and Betsholtz 2003), however, these markers are not specific to, nor recognize, all pericytes (Armulik et al. 2005). Pericyte recruitment to the vessel wall is essential for the maturation, stability and functionality of vessels (Benjamin et al. 1998). Pericytes stimulate endothelial cell basement membrane formation (Stratman et al. 2009) and control perfusion (Carmeliet and Jain 2011). Mural cells can be derived from bone marrow (Carmeliet 2000), adipose tissue stroma (Traktuev et al. 2008; Wang et al. 2010), and from fibroblasts that can differentiate into myofibroblasts and further into vascular smooth muscle cells (Chambers et al. 2003).

Four pathways are involved in vascular wall maturation: i) PDGF-B – PDGFR β . PDGF-B secretion by endothelial cells induces recruitment of PDGFR β expressing mural cells. ii) sphingosine -1-phosphate-1 (SIP -1) – endothelial differentiation sphingolipid G-protein-coupled receptor -1 (EDG1). SIP-1 induces the recruitment of EDG1 expressing mural cells. iii) Ang-1 - tyrosine kinase (T) with Ig (I) and epidermal (E) growth factor homology domain 2 (Tie-2) pathway. Pericytes and vascular smooth muscle cells express Ang-1, and they bind tightly to the their receptor Tie-2 on the surface of endothelial cells. This interaction mediates blood vessel stabilization. iv) TGF- β . Ang-1/Tie-2 contact induces activation of TGF- β , which further induces basement membrane formation. (Carmeliet and Jain 2011; Holderfield and Hughes 2008; Jain 2003)

The re-established contact between endothelial cells and pericytes induces expression of TIMP-2 in endothelial cells and TIMP-3 in pericytes, which switches off the proteolytic phenotype of endothelial cells (Saunders et al. 2006). TIMPs and plasminogen activator inhibitor 1 (PAI-1) induce the deposition of basement membrane (Carmeliet and Jain 2011). The basement membrane is built up of laminins, collagen IV (COL IV), perlecan, nidogens and COL XVIII (Davis and Senger 2005). Basement membrane keeps endothelial cells apart from COL I, which is needed in order to prevent the activation of endothelial cells. The adult endothelial cells are therefore stable mainly due to basement membrane. After

establishment of blood flow, the local oxygen levels increase which leads to the decrease in VEGF levels and the end of angiogenesis. (Darland and D'Amore 1999)

Maturation of the vascular network involves optimal patterning of the network by branching, expanding and pruning (Jain 2003). Some of the capillaries remain, while some of the capillaries differentiate into arteries or veins (Risau 1997). Increase in shear stress and pressure induces recruitment of smooth muscle cells into capillaries, leading to differentiation into arteries or veins (Buschmann and Schaper 1999). Expansion of larger arteries and veins acquires several layers of mural cells in addition to ECM and elastic laminae. This is essential to obtain viscoelastic properties and neural control for the blood vessels. (Jain 2003) Endothelial cells, mural cells and matrix undergo also organ-specific specialization.

Role of extracellular matrix in angiogenesis

ECM provides support for existing and developing vasculature and guidance for the new forming capillaries. ECM stores biologically active molecules such as angiogenesis inducers and inhibitors. (Carmeliet and Jain 2011) Many ECM proteins such as COL I, III and XV, collagen receptor integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, laminin-1 and -8, fibronectin, fibronectin receptor integrin $\alpha_5\beta_1$ as well as perlecan have angiogenic properties (Jain 2003). Moreover, several angiogenic factors are bound in heparin sulphate in the ECM (e.g. VEGF, bFGF and TGF β) (Rundhaug 2003). ECM components thrombospondin 1 and 2 (Tsp-1 and Tsp-2, respectively) inhibit angiogenesis (Jain 2003). Many other angiogenesis inhibitors such as endostatin (derived from COL XVIII) (O'Reilly et al. 1997), angiostatin (derived from plasminogen) (Dong et al. 1997), as well as tumstatin (derived from COL IV) (Hamano et al. 2003), are also stored in ECM as fragments within larger matrix molecules.

The balance in protease activity and interactions of proteases with ECM regulate angiogenesis (Christiaens and Lijnen 2010). During angiogenesis and vessel remodeling, the proteolytic modification of ECM allows endothelial cells to migrate and converts the basement membrane into a proangiogenic environment (Carmeliet and Jain 2011; Lamalice et al. 2007). Three classes of proteases; serine proteases, cysteine proteases, and metalloproteases participate in the ECM remodeling (Christiaens and Lijnen 2010). These proteases generate angiogenic and anti-angiogenic factors from ECM proteins and modify growth factors and receptors (van Hinsbergh et al. 2006). MMPs promote angiogenesis by degrading basement membrane and by regulating endothelial cell attachment, proliferation and migration as well as by releasing growth factors from ECM (Stetler-Stevenson 1999). One such MMP, cathepsin B, having both angiogenic and anti-angiogenic effects, is suggested to be the "angiogenic

switch" of endothelial cells (Christiaens and Lijnen 2010; Im et al. 2005). If no blood flow is created, the capillaries are repressed by MMPs (Buschmann and Schaper 1999; Davis and Saunders 2006).

The fibrinolytic system also participates in angiogenesis, in wound healing and in tissue remodeling (Christiaens and Lijnen 2010). Plasminogen is an inactive proenzyme that, when converted into the active enzyme, plasmin, degrades fibrin (Christiaens and Lijnen 2010). Plasmin activates and releases MMPs, elastase and growth factors such as VEGF, bFGF, HGF, TGF β and PDGF (Tkachuk et al. 2009). Two plasminogen activators are tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) (Christiaens and Lijnen 2010). uPA stimulates endothelial cell tubule formation (Christiaens and Lijnen 2010; Tkachuk et al. 2009). Plasminogen activator inhibitor 1 (PAI-1) is the principal inhibitor of uPA and tPA, and has a significant role in the regulation of ECM remodeling (Christiaens and Lijnen 2010). ECM associated proteases PAI-1 and uPA are activated by VEGF (Pepper 2001).

Molecular regulation of angiogenesis

Angiogenesis is tightly regulated by several positive and negative regulators; cells, soluble factors and extracellular matrix components (Fan et al. 1995). Some of the most important factors are next discussed in detail.

The Vascular Endothelial Growth Factor family

The VEGF protein family contains VEGF (VEGF-A), VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF) (Christiaens and Lijnen 2010). VEGF is the predominant and most potent inducer of angiogenesis, inducing endothelial cell proliferation, sprouting and tubule formation (Otrock et al. 2007), both in normal and in tumor angiogenesis (Carmeliet and Jain 2011). Three forms of VEGF are produced in alternative splicing (VEGF-A121, VEGF-A165, VEGF-A189) (Christiaens and Lijnen 2010). Paracrine VEGF, secreted by tumor, myeloid or other stromal cells increases vessel enlargement and branching (Stockmann et al. 2008) whereas autocrine VEGF, released by endothelial cells, maintains vessel homeostasis (Lee et al. 2007). Soluble VEGF isoforms enlarge vessels, whereas membrane-bound VEGF induces vessel branching (Iruela-Arispe and Davis 2009).

All the members of VEGF family signal through three transmembrane tyrosine kinase receptors, VEGF receptor-1 (VEGFR-1, also known as Flt-1), VEGFR-2 (also known as Flk-1) and VEGFR-3 (Ferrara 2004). VEGF interacts through VEGFR-1 (Christiaens and Lijnen 2010) and VEGFR-2 (Ferrara 2009; Nagy et al. 2007). VEGF-B and PlGF bind to VEGFR-1

(Hagberg et al. 2010). VEGF-C acts through VEGFR-2 and VEGFR-3 (Tvorogov et al. 2010) and VEGF-D through VEGFR-3 (Stacker et al. 2001). VEGFR-1 and VEGFR-2 mediate angiogenesis, whereas VEGFR-3 is known to be important in embryogenesis and adult lymphangiogenesis (Tammela and Alitalo 2010; Veikkola et al. 2001).

VEGF-B is 43% identical to VEGF-A165 (Christiaens and Lijnen 2010). VEGF-B has restricted angiogenic activity in certain tissues, such as heart (Hagberg et al. 2010). VEGF-B also participates in ECM degradation (Olofsson et al. 1998). VEGF-C that activates tip cells (Tvorogov et al. 2010), is 30% identical to VEGF-A165 (Christiaens and Lijnen 2010). VEGF-D is 48% identical to VEGF-C (Christiaens and Lijnen 2010). It promotes lymphangiogenesis (Stacker et al. 2001). PlGF is 43% identical to VEGF-A165 (Christiaens and Lijnen 2010). PlGF is not relevant for developmental angiogenesis, but has a role as an angiogenic factor in pathological conditions (Carmeliet et al. 2001; Christiaens and Lijnen 2010). PlGF has been shown to increase revascularization in ischemic tissue, in wounded skin and in tumors (Carmeliet and Jain 2011).

Platelet Derived Growth Factor- β

PDGF- β induces proliferation of smooth muscle cells and fibroblasts *in vitro* (Kiritsy et al. 1993). PDGF is a homodimer of two polypeptides B (PDGF-BB). PDGF polypeptides can also assemble into other hetero- or homodimers (PDGF-AA, -CC, -DD, or PDGF-AB). PDGF receptors (PDGFR) are also assembled into dimers, PDGFR- $\alpha\alpha$, PDGFR- $\beta\beta$ and PDGFR- $\alpha\beta$. PDGF- β is the only PDGF isoform that can bind all these receptor dimers. (Wang et al. 2012) Angiogenic endothelial cells release PDGF-B to chemoattract PDGFR- β expressing pericytes (Hellberg et al. 2010). The interaction between PDGF- β and PDGFR- β is the key for the pericyte recruitment and the development of functional vasculature (Hellstrom et al. 1999). PDGF has also an important role in wound healing (Kiritsy et al. 1993).

The Ang/Tie signaling

Ang/Tie signaling provides a maintenance and adjustment system for normal healthy vessels (Carmeliet and Jain 2011). The signaling system consists of two receptors, Tie-1 and Tie-2, expressed by endothelial cells and three ligands, Ang-1, Ang-2 and Ang-3 (Christiaens and Lijnen 2010). Ang-1 is expressed by mural and tumor cells, whereas Ang-2 is expressed by angiogenic tip cells (Christiaens and Lijnen 2010; Jain 2003). Tie-2 binds both Ang-1 and Ang-2, but the ligands of Tie-1 are less well-known (Christiaens and Lijnen 2010). Ang-2 either activates or blocks Tie-2, depending on cells, whereas Ang-1 constantly activates Tie-2 (Davis et al.

1996). Ang-1 tightens vessels by recruiting pericytes and promoting interaction between endothelial cells and mural cells (Carlson et al. 2001). Ang-1 is also reported to inhibit inflammation under normal physiological conditions (Thurston et al. 1999). Ang-2 stimulates vessel growth by destabilizing endothelial cell-pericyte interactions, dissociating pericytes from vessels and by degrading ECM (Cao et al. 2007; Morange et al. 2002).

Transforming Growth Factor- β

TGF- β is a multifunctional cytokine that regulates growth and differentiation and is produced by a variety of cells (Christiaens and Lijnen 2010). Ang-1/Tie-2 contact induces secretion of TGF- β (Holderfield and Hughes 2008). TGF- β is secreted by macrophages and other stromal vascular fraction cells in adipose tissue (Bourlier et al. 2008). It has both pro- and antiangiogenic properties. At low levels it upregulates angiogenic factors and ECM degrading proteases, whereas at high levels it inhibits endothelial proliferation and promotes tubule maturation by inducing basement membrane formation and differentiation of mesenchymal cells into mural cells (Holderfield and Hughes 2008; Pardali et al. 2010).

basic Fibroblast Growth Factor

bFGF belongs to a superfamily of FGFs, the widely expressed mitogens that control a number of biological functions (Beenken and Mohammadi 2009). FGFs are needed to maintain vascular integrity (Murakami et al. 2008). FGFs also indirectly stimulate angiogenesis by activating other angiogenic factors like interleukin 6 (IL-6) (Beenken and Mohammadi 2009; Okamura et al. 1991). bFGF was one of the first angiogenic factors discovered (Carmeliet and Jain 2011). bFGF stimulates endothelial cell proliferation, migration and differentiation by activating its receptor on endothelial cells (Kawaguchi et al. 1998). When bFGF is bound to its tyrosine kinase receptors FGFR1 and FGFR2, it activates downstream MAPK signaling and induces cell proliferation (Cross and Claesson-Welsh 2001). bFGF also stimulates the synthesis of proteases, such as collagenase, during angiogenesis (Okamura et al. 1991).

A schematic drawing of angiogenesis and its regulators is seen in Figure 1.

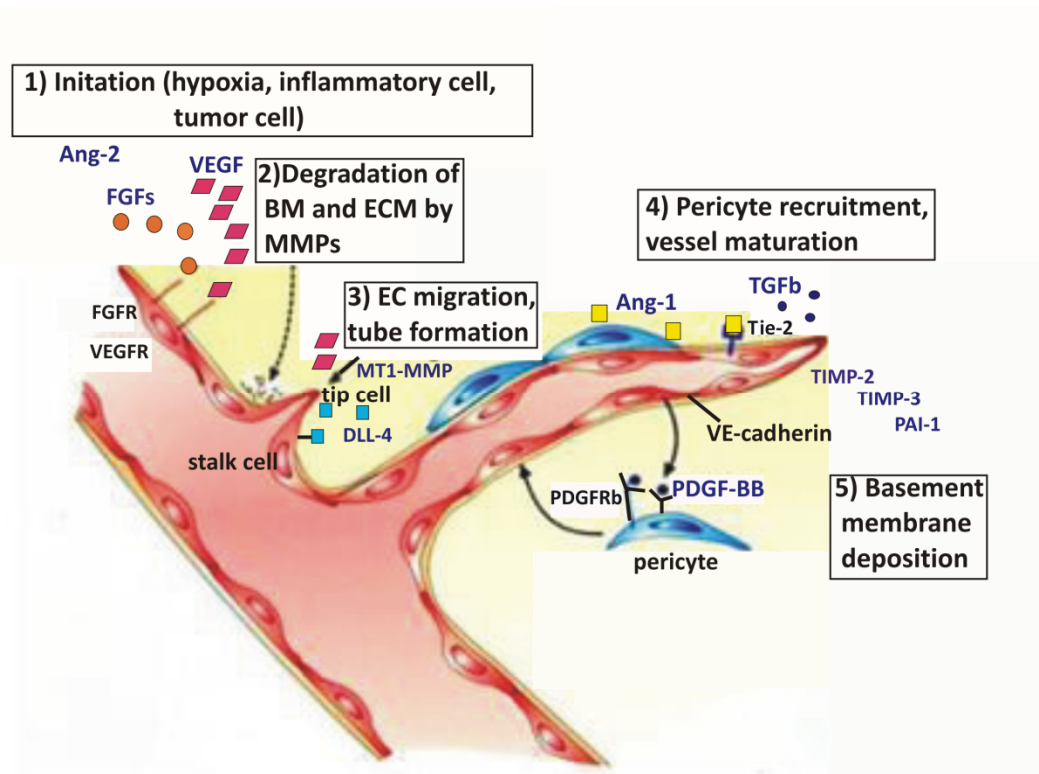


Figure 1. Angiogenesis and its main regulators. Angiogenesis is first initiated by growth factor secretion (e.g. Ang-2, FGFs, VEGF) from hypoxic cells, inflammatory cells or tumor cells. Growth factors are attached to their receptors in endothelium, which activates the endothelial cells and induces the degradation of basement membrane and ECM by MMPs. Endothelial tip cell then migrates towards the VEGF gradient. Tip cell secretes MT1-MMP, which degrades ECM, and DLL-4, which binds to its receptor Notch in stalk cells. After tube formation and elongation, PDGFRβ expressing pericytes are recruited by PDGF-B expressing endothelial cells. Ang-1 expressing pericytes bind tightly to their receptor Tie-2, which induces TGFβ production from endothelial cells. After basement membrane deposition, endothelial cells become surrounded by COL IV and obtain their quiescent state. Image modified from Klagsbrun and Moses (1999).

Angiogenesis in tissue repair

Neovascularization is a critical step in tissue repair such as in wound healing. Tissue injury causes disruption of blood vessels and the release of blood constituents into wound site. During the early phase of wound repair, granulation tissue appears. At first, neutrophils cleanse the wound from foreign particles and bacteria. Activated platelets stimulate the formation of hemostatic plug, as well as the release of growth factors such as PDGF, that then attract and activate macrophages and fibroblasts. Hypoxia induces secretion of VEGF. (Epstein 1999; Tonnesen et al. 2000) Macrophages and the secreted factors are essentially needed for the new

tissue formation (Epstein 1999). Peripheral blood monocytes are recruited into the injury site by ECM fragments as well as by TGF β and monocyte chemotactic protein-1 (MCP-1). Monocytes are then activated into macrophages which ensure the continuous synthesis and secretion of growth factors (e.g. PDGF and VEGF) for the formation of granulation tissue. (Epstein 1999; Tonnesen et al. 2000) Macrophages also secrete CSF-1, TNF α , TGF α , TGF β , IL-1, and IGF-1 (Rappolee et al. 1988). Chemotactic factors can also be secreted by parenchymal cells. They may trigger release of bFGF and aFGF during the first three days of wound healing. This is the initiation stimulus of angiogenesis in wound healing. (Tonnesen et al. 2000) bFGF stimulates endothelial cells to release plasminogen activator and procollagenase (Magnatti et al. 1989). Plasminogen activator converts plasminogen into active plasmin and procollagenase into active collagenase that then degrade basement membrane allowing endothelial sprouting. Endothelial cells migrate in response to the angiogenic factors such as VEGF. Integrin $\alpha v \beta 3$ is suggested to be critical for wound repair angiogenesis. The tips of sprouting endothelial cells express $\alpha v \beta 3$, which is needed to the endothelial cell migration into fibrin/fibronectin-rich provisional ECM in the wound clot. The provisional matrix is later replaced by collagen-rich scar tissue, and majority of the neovasculature undergoes apoptosis. (Tonnesen et al. 2000)

Perfusion independent role of endothelial cells

Endothelial cells and endothelial cell secreted factors have an important perfusion independent role in tissue development and remodeling. In embryogenesis, the endothelial cell migration into developing organs provides inductive signals to promote organogenesis. This does not require blood flow or conduits for oxygen delivery, but instead, the cells produce stimulating factors, so called angiocrine factors, that affect paracrinically organogenesis and tissue remodeling. (Butler et al. 2010; Ding et al. 2010) Angiocrine factors are, for example, growth factors, cytokines or adhesion molecules like bFGF, VEGF-A, Ang-2, PDGF-B, bone morphogenic protein-2 and -4 (BMP-2 and -4, respectively), MCP-1, intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1), IL-6 and IL-8 that can enhance angiogenesis and tissue repair (Butler et al. 2010). During development, endothelial cells secrete inductive signals that promote organ development, even in the absence of blood flow (Butler et al. 2010; Lammert et al. 2001). Endothelial cells can therefore support the maintenance and induction of tissue stem and progenitor cells (Butler et al. 2010).

Adipose tissue

The human body has two main types of adipose tissue, brown and white adipose tissue (Choi et al. 2010). Brown adipose tissue is a major type of adipose tissue in prenatal period (Bucky and Percec 2008). In adult, brown adipose tissue is mainly found in neck, mediastinum and supraclavicular areas (Nedergaard et al. 2007). Brown adipose tissue is mainly used for heat generation from triglycerides (Bucky and Percec 2008), and it is activated during cold exposure (Nedergaard et al. 2007).

White adipose tissue is a primary energy storage site in the body. It is distributed as depots in hypodermis (subcutaneous fat), around internal organs (visceral fat) and in other sites such as in bone marrow, in pericardium and in breast (REF). Adipose tissue stores energy as triglycerides in adipocytes, and rapidly releases them when needed (Rosen and Spiegelman 2006). Adipose tissue is a unique organ, as it has a capacity to undergo continuous expansion and regression throughout adult life (Sun et al. 2011; Poulos et al. 2010). Subcutaneous adipose tissue deposits are the main adipose tissue depots in humans (Harrington et al. 2004). Visceral adipose tissue i.e. internal adipose tissue deposits are known to be more active metabolically than subcutaneous adipose tissue (Fain et al. 2004).

Adipose tissue is composed of mature adipocytes surrounded by stromal-vascular fraction (SVF) cells. SVF is a heterogenous population of cells containing fibroblasts, mast cells, macrophages, leukocytes, endothelial cells, pericytes, adipose stem cells and ECM components (Astori et al. 2007; Karastergiou and Mohamed-Ali 2010; Poulos et al. 2010). 50% of adipose tissue cells are mature adipocytes, 10% resident macrophages and the rest comprises of other stromal vascular cells (Karasterigou and Mohamed-Ali 2010). Adipose tissue is very rich in blood vessels, at least one capillary surrounding each adipocyte (Lijnen 2008), and also innervated by sympathetic nervous system (Bartness and Bamshad 1998). ECM interconnects adipocytes and forms the fat lobules in adipose tissue (Bucky and Percec 2008).

Adipocytes

Adipocytes are specialized connective tissue cells that have a basic function to store energy as triglycerides in lipogenesis and to release them as fatty acids in lipolysis. Adipocytes release a wide number of proteins and peptides that act in endocrine, paracrine or autocrine manner. They are implicated as adipokines. The most abundant adipokines released by adipocytes are leptin and adiponectin. (Galic et al. 2010; Karastergiou and Mohamed-Ali 2010)

Adipose stromal cells

Multiple cells in adipose tissue share a common progenitor, adipose stromal cell (ASC), also widely known as adipose stem cell. Adipose stromal cell is a name suggested by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy to be used of these plastic-adherent stromal vascular fraction cells, rather than stem cells (Dominici et al. 2006).

ASC are defined as a population of cells in SVF that differentiate into adipocytes, chondroblasts and osteoblasts *in vitro* (Dominici et al. 2006). Moreover, ASC express cell surface markers CD105 (endoglin), CD90 (Thy-1) and CD73 (Dominici et al. 2006). ASC also typically express CD13 (aminopeptidase N), CD29 (integrin β 1), CD44 (hyaluronate), CD71 and CD10 (CALLA/neutral endopeptidase) (Lindroos et al. 2010; Lindroos et al. 2009; Zuk et al. 2002) as well as CD34 (Lindroos et al. 2010; Zimmerlin et al. 2010).

ASC are known to have developmental plasticity both *in vitro* and *in vivo* (Planat-Benard et al. 2004; Rehman et al. 2004). They have capacity to differentiate into multiple cell phenotypes such as adipose tissue cells (Gimble and Guilak 2003; Zuk et al. 2001), contractile smooth muscle cells (Traktuev et al. 2008), skeletal muscle cells (Di Rocco et al. 2006; Zuk et al. 2001), cartilage (Awad et al. 2004), bone (Zuk et al. 2001) endothelial cells (Miranville et al. 2004; Oswald et al. 2004; Pittenger et al. 1999; Planat-Benard et al. 2004; Wosnitza et al. 2007; Wu et al. 2007) and neuronal cells (Ning et al. 2006). ASC are known to promote vessel growth, maturation and stabilization *in vivo* (Amos et al. 2008; Cai et al. 2009; Covas et al. 2008; Traktuev et al. 2008; Zannettino et al. 2007) by secreting angiogenic factors such as VEGF, HGF, TGF β , IL-6, IL-8 and by differentiating into vessel lining cells with pericytic properties (Amos et al. 2008; Kilroy et al. 2007; Merfeld-Clauss et al. 2010; Miranville et al. 2004; Traktuev et al. 2008).

The determination of ASC phenotype is strongly regulated by the tissue microenvironment (Balwierz et al. 2008; Stacey et al. 2009). ASC are reported to play a role in host defense (Saillan-Barreau et al. 2003) and they are able to modulate the inflammatory profile of macrophages *in vitro* into anti-inflammatory phenotype (Hanson et al. 2011) and to suppress inflammatory response (Gonzalez-Rey et al. 2010).

In adipose tissue, a subpopulation of perivascular cells serves as adipocyte progenitor cells (Traktuev et al. 2008; Zannettino et al. 2007). These specific cells contribute to vessel maturation by co-operating with endothelium during blood vessel formation (Traktuev et al. 2008) and as pericytes in blood vessel wall of adipose tissue (Cai et al. 2011; Tang et al. 2008). It suggested that ASC are resided within the pericytic population surrounding the blood vessels in adipose tissue and that these cells contain pluripotent adipose stem cells (Cai et al. 2011). However, only a limited

number of pericytes and adipose tissue stroma are adipose stem cells (Cai et al. 2011; Zannettino et al. 2008).

Adipogenesis

Adipogenesis begins with the appearance of several fat clusters called primitive organs. These are vascular structures with few or no fat cells. (Christiaens and Lijnen 2010) The adipocyte differentiation proceeds with mesodermal cell differentiation into adipoblasts and further into ASC, then into committed preadipocytes and finally into mature lipid-synthesizing and sorting adipocytes (Bucky and Percec 2008). Adipose tissue can be expanded by adipocyte hypertrophy, where the existing cells grow in size, or by hyperplasia, increase in adipocyte number, which requires progenitor cells to differentiate into adipocytes (Spalding et al. 2008).

Glucose and free fatty acids are the main molecules that lead to the synthesis of triglycerides (Bederman et al. 2009). Growth arrest of the preadipocyte is required for the adipocyte differentiation. In the early phase of adipogenesis, the ASC change shape from fibroblast-like shape into more spherical shape. (Gregoire 2001) Simultaneously ECM is degraded and cytoskeletal components are modified (Gregoire et al. 1998; Selvarajan et al. 2001). ECM modifications are required for the key gene activation and lipid accumulation into cells (Selvarajan et al. 2001; Gregoire 2001). The number of mitochondria is also largely increased during adipogenesis (Wilson-Fritch et al. 2003) as a response to the increased metabolic demand (Wilson-Fritch et al. 2004).

To obtain the mature adipocyte, activation of over 2000 genes is required (Guo and Liao 2000). The most important transcriptional factors involved in adipogenesis are CCAAT/enhancer binding proteins (C/EBP) α , β and δ and peroxisome proliferator activated receptor γ (PPAR γ), that is activated by C/EBPs (Farmer 2006; Rosen and Spiegelman 2000). PPAR γ , the main regulator of adipogenesis, is activated early in adipogenesis (specifically PPAR γ 2 in adipose tissue) by C/EBP β and C/EBP δ (Rosen and Spiegelman 2000; Gregoire 2001). Activation of PPAR γ is the key switch for adipocyte differentiation (Bucky and Percec 2008; Daquinag et al. 2011; Lowe et al. 2011). C/EBP β increases expression of another key inducer of angiogenesis, C/EBP α (Wu et al. 1996). PPAR γ also further activates C/EBP α by positive feedback signal to maintain the differentiated state (Daquinag et al. 2011). In addition, cyclic adenosine monophosphate (cAMP) responsive element binding protein (CREB) is activated and expressed prior to and during adipogenesis (Gregoire 2001). Lipoprotein lipase (LPL), Kruppel-like transcription factor 5 (KLF5) (Oishi et al. 2005), early growth response 2 (Krox20) (Chen et al. 2005) early B-cell (O/E-1) factor (Akerblad et al. 2002), glucocorticoids, prostacyclin (PGI₂) as well as activation of the MEK/ERK and the p38 MAPK signalling pathways are required for adipogenesis (Engelman et al. 1999; Prusty et al. 2002).

C/EBP β and C/EBP δ activate KLF5 and they in concert then activate PPAR γ 2 (Oishi et al. 2005). PGI₂, released from preadipocytes, activates the protein kinase A (PKA) pathway (Vassaux et al. 1992) and upregulates C/EBP β and C/EBP δ (Belmonte et al. 2001). Insulin and insulin-like growth factor -1 (IGF-1) are also required for adipocyte differentiation (Bucky and Percec 2008). Insulin and lipids activate sterol-regulatory element binding protein 1c (SREBP1c) that also induces PPAR γ (Daquinag et al. 2011). SREBP1c further activates a variety of genes, that along with PPAR γ and C/EBP α activate late adipogenesis marker proteins such as fatty acid synthetase (FAS), insulin-regulated glucose transporter 4 (GLUT4), adipsin, angiotensinogen II, acyl-coenzyme A – binding protein (ACBP), fatty acid binding protein (aP2), keratinocyte lipid-binding protein (KLBP), lipoprotein lipase (LPL), *sn*-1-acylglycerol-3-phosphate acyltransferase2 (AGPAT2) and perilipin (Bucky and Percec 2008; Daquinag 2011; Lowe et al. 2011). At the terminal phase of differentiation, enzymes of triacylglycerol synthesis and degradation are activated. Glucose transporters, insulin receptors and insulin sensitivity are also increased. Synthesis of adipocyte-secreted products including leptin, adipsin, resistin, and adipocyte-complement-related protein (Acrp30, adiponectin) begin. (Gregoire 2001) Mature adipocytes secrete also COL IV, laminin, entactin and glycosaminoglycans (Bucky and Percec 2008).

Several inflammatory cytokines, including tumour necrosis factor- α (TNF- α), TGF- β , IL-1, IL-6, IL-11, leukaemia inhibitory factor (LIF), interferon- γ , oncostatin M and ciliary neurotrophic factor (CNTF), can inhibit stem cell differentiation (Gimble et al. 1989; Gimble et al. 1994) or even induce stem cell dedifferentiation (Ron et al. 1992). However, LIF has also reported to have stimulating or diverse effects on angiogenesis (Hogan and Stephens 2005). The activity of PPAR γ is regulated by TNF- α (Ye 2008). Growth hormone (GH) represses adipogenesis by inhibiting C/EBP α and PPAR- γ (Ross et al. 2000). GATA-binding transcription factors GATA-2 and -3 as well as preadipocyte factor -1 (Pref-1) expressions are high in ASC, and their downregulation is required for adipogenesis (Bucky and Percec 2008; Feve 2005; Gregoire 2001).

A schematic drawing of adipogenesis and its main transcriptional regulators is shown in Figure 2.

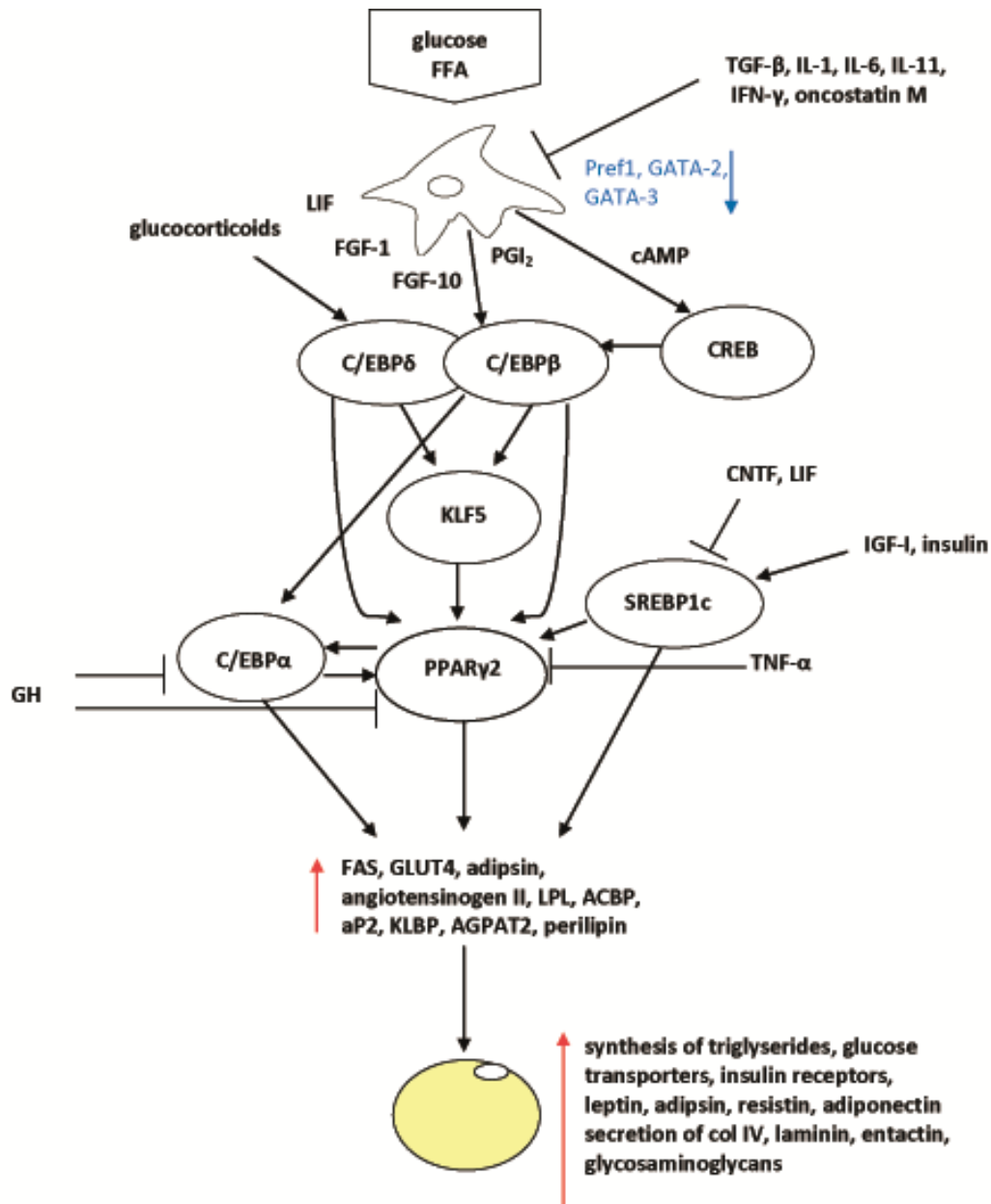


Figure 2. Adipogenesis and its main regulators. Glucose and free fatty acids are the main triggers for adipogenesis. During the first steps of adipogenesis, expression of Pref-1 is decreased in ASC. The main transcriptional factors are C/EBPs and PPAR γ , which is activated by C/EBPs. PPAR γ activates C/EBP α by positive feedback signaling. Glucocorticoids, FGFs, LIF, insulin, IGF-I and PGI₂ induce adipogenesis. SREBP1c activates several genes that along with PPAR γ and C/EBP α activate late adipogenesis markers such as ACBP and aP2. Synthesis of adipocyte secreted products, such as leptin and adiponectin, and basement membrane components such as COL IV, begin.

Adipose tissue angiogenesis

Blood vessel growth is essential for every organ, including the orderly development of adipose tissue. In developing embryo, the vascular system differentiation is developed prior to the adipogenesis (Hausman and Richardson 2004; Crandall et al. 1997).

During adipogenesis, angiogenic vessels contribute to adipose tissue expansion by supplying nutrients and oxygen to tissue and by removing waste (Cao 2007; Cao 2010). Importantly, vessels supply the tissue with growth factors and cytokines as well as with circulating stem/progenitor cells which have the capacity to differentiate into adipose tissue cells (Tang et al. 2008). Also monocytes and neutrophils are infiltrated from bone marrow into adipose tissue through vasculature (Tang et al. 2008; Cao 2010).

Adipose tissue expansion and function require parallel growth and remodeling of the capillary network (Christiaens and Lijnen 2010). Expansion of adipose tissue can be promoted by either neovascularization or by dilation and remodeling of the existing capillaries (Hausman and Kaufmann 1986). The regulation of angiogenesis in adipose tissue is dependent on the local balance between pro- and antiangiogenic growth factors and cytokines (Christiaens and Lijnen 2010; Cao 2010). Activated endothelial cells communicate with adipocytes and secrete growth factors and cytokines, and vice versa (Cao 2007; Cao 2010). Many of the inductive angiogenic factors are derived from adipose tissue cells (Crandall et al. 1997; Hausman and Richardson 2004; Planat-Benard et al. 2004; Saiki et al. 2006). Vessels determine both the local and systemic effects of the adipose tissue secreted factors (Cao 2010). The adipose tissue secretory products and their functions in angiogenesis induction are next explained in detail.

Adipose tissue secretory products

Adipose tissue is a major source of growth and differentiation promoting factors in body (Kershaw and Flier 2004; Kilroy et al. 2007; Rehman et al. 2003; Rehman et al. 2004; Traktuev et al. 2008; Trayhurn 2005; Trayhurn and Beattie 2001). Mature adipose tissue secretes numerous hormones, growth factors, matrix proteins, enzymes, proinflammatory and anti-inflammatory cytokines as well as coagulation and complement factors (Kershaw and Flier, 2004; Kilroy et al., 2007; Poulos et al. 2010; Rehman et al., 2003; Rehman et al., 2004; Traktuev et al., 2008; Trayhurn, 2005; Trayhurn and Beattie, 2001). The secreted factors participate in the regulation of adipocyte differentiation, fat mass accumulation and remodeling, in the development of vasculature and blood flow as well as function of immune system (Poulos et al. 2010). The major organ systems

affected by adipose tissue derived factors are vasculature, liver, muscle, pancreas β cells, brain and reproductive tract (Scherer 2006). There are detailed studies where the adipose tissue secreted factors have been characterized (Alvarez-Llamas et al. 2007; Fain et al. 2004). Most of the adipose tissue secreted factors are derived from stromal vascular cells. Visceral adipose tissue is known to secrete more factors than subcutaneous tissue, for example, 400% greater amount of VEGF is released from visceral adipose tissue than from subcutaneous adipose tissue. (Fain et al. 2004)

The main endogenous factors released from adipose tissue and their effect on adipogenesis and angiogenesis as reported in the main references are summarized in Table I. Some of the important factors are also discussed in detail below. A few of the secretory products of adipose tissue related to angiogenesis induction (VEGF, PDGF-BB, TGF- β , bFGF, Angiopoietins) are discussed in detail earlier in chapter "Molecular regulation of angiogenesis". In addition to the ones presented here, adipose tissue secretes and synthesizes other factors and adhesion molecules as well as plasma membrane and nuclear receptors.

Adipokines

The most abundant endocrine hormones released by adipocytes are leptin and adiponectin. Leptin and adiponectin have opposite functions in hypothalamus, and they regulate the balance between energy storage and uptake (Galic et al. 2010).

Leptin

Leptin is one of the major adipogenic and angiogenic hormone (Karastergiou and Mohamed-Ali 2010; Galic et al. 2010; Christiaens and Lijnen 2010). Leptin is produced by mature adipocytes, mainly from subcutaneous adipose tissue (Christiaens and Lijnen 2010). Leptin is also secreted from stomach (Cinti et al. 2000), placenta and fetal tissues (Cervero et al. 2006). Leptin has a key role in regulating energy balance. It functions as a signal of negative energy balance and low energy stores. In lean (or normal) people, high leptin levels reduce food intake and fat storage, however, although leptin levels are elevated in obese individuals (Wang et al. 2008), obese persons are resistant to leptin and continue to maintain high levels of body fat (Galic et al. 2010). Leptin stimulates angiogenesis by inducing migration of endothelial cells and by promoting the expression of VEGF and VEGFR-2 (Suganami et al. 2004). Leptin also possibly induces cytokine-related signaling pathways e.g. IL-6, CNTF and LIF pathways (Ailhaud 2006). Leptin signals through receptor LR (also called as obR). Alternative splicing of the leptin gene results in at least six receptor isoforms (LRa - LRf) (Sweeney 2002) that can be divided into

three classes: long, short and secreted (soluble) isoforms (Bates and Myers Jr 2003). The long isoform LRb is expressed mainly in hypothalamus and regulates body weight and feeding (Ahima and Flier 2000; Tartaglia 1997). The long isoform is assumed to mediate most of the leptin signaling (Sweeney 2002). In fact, the lack of leptin signaling, especially the long isoform, results in obesity in such individuals (Lee et al. 1996). Leptin has receptor isoforms in almost all other tissues as well, such as in heart, placenta, lung, liver, kidney, small intestine, ovary, pancreas, spleen and skeletal muscle (Ahima and Flier 2000; Dulloo et al. 2002; Kielar et al. 1998; Tartaglia et al. 1995). The short isoforms are suggested to mediate leptin transport through blood-brain barrier and regulate leptin degradation (Banks et al. 1996). The secreted isoform regulates concentration of free leptin in blood (Ge et al. 2002).

Adiponectin

Adiponectin, (also known as an adipocyte complement-related protein 30, Arcp 30) a major adipogenic hormone, is an abundant circulating plasma protein (Christiaens and Lijnen 2010; Hu et al. 1996). Adiponectin is exclusively secreted by mature adipocytes (Hu et al. 1996; Wang et al. 2008). It is mainly known as an inhibitor of angiogenesis (Brakenhielm et al. 2004). However, it is reported to have a dual role and act also as a stimulator of angiogenesis (Ouchi et al. 2004; Shibata et al. 2004). Adiponectin is highly expressed in lean people, but obesity reduces adiponectin levels (Matsubara et al. 2002; Ouchi et al. 1999). Adiponectin improves insulin sensitivity and inhibits glucose secretion from liver (Combs et al. 2001; Ouchi et al. 2001). PPAR γ ligands have shown to increase adiponectin expression and plasma concentration *in vitro* and *in vivo* (Maeda et al. 2001).

Cytokines

Cytokines are peptides that are bioactive at very low levels and play an integral role in the regulation of inflammation, cell proliferation and maturation. Over 100 cytokines have been described and classified as interleukins, interferons, chemokines, haematopoietic factors and growth factors. (Thalmann and Meier 2007) Chemokines are small secreted basic proteins that are implicated in the chemoattraction of inflammatory cells (Juge-Aubry et al. 2005).

Adipose tissue secretes a wide number of cytokines such as TNF α , IL-1 β , IL-6, IL-8, IL-10, IL-11, LIF, interferon- γ , oncostatin M, CNTF, CC-chemokine 5 (CCL5), MCP-1, visfatin, vaspin and omentin. The majority of interleukins and other inflammatory cytokines in human adipose tissue are released from SVF cells (Fain 2006; Weisberg et al. 2003). IL-6 plasma

levels are increased in type 2 diabetes and obesity (Lazar 2005). TNF- α is a multifunctional cytokine with several immunological functions. It is mainly secreted from macrophages and it promotes endothelial cell tubule formation (Papetti and Herman 2002). It also promotes obesity-induced insulin resistance (Christiaens and Lijnen 2010; Galic et al. 2010). The increased expression of TNF- α in obesity is due to infiltration of circulating macrophages (Weisberg et al. 2003).

Angiogenin induces angiogenesis by activating vessel endothelial and smooth muscle cells and triggering a number of biological processes, including cell migration, invasion, proliferation, and formation of tubular structures. Angiogenin is secreted by mature adipocytes and stromal vascular fraction cells. (Gao and Xu 2008)

Growth factors

The two main angiogenic growth factors secreted by adipose tissue are VEGF and hepatocyte growth factor (HGF). Adipose tissue secretes also several other growth factors such as bFGF, FGF-1, FGF-9, insulin-like growth factor I (IGF-I), VEGF-B, VEGF-C, PlGF, PDGF- β , Ang-1 and Ang-2. (Cao 2007; Wang et al. 2008; Cao 2010)

VEGF is expressed by stromal vascular cells and mature adipocytes (Ledoux et al. 2008; Voros et al. 2005). VEGF is highly expressed in adipose tissue and the expression is enhanced during adipocyte differentiation (Claffey et al. 1992). VEGF is thought to be responsible for the most of the angiogenic capacity of adipose tissue (Zhang et al. 1997). The VEGF functions in angiogenesis are discussed in detail earlier. HGF is a molecule with multifunctions, derived from mesenchymal cells (Christiaens and Lijnen 2010) such as fibroblasts, hepatic cells or adipose stromal cells (Traktuev et al. 2008). HGF promotes tubule formation of HUVEC (Saiki et al. 2006) and has a central role in regulating angiogenesis in developing adipose tissue (Bell et al. 2008). HGF concentrations are elevated in obese individuals (Rehman et al. 2003). bFGF is a multifunctional proliferative growth factor which functions are discussed in detail earlier. bFGF promotes angiogenesis and induces adipocyte differentiation *in vivo* (Kawaguchi et al. 1998). bFGF also induces HGF secretion in ASC (Kilroy et al. 2007). Macrophage derived PDGF stimulates angiogenesis during expansion of adipose tissue (Pang et al. 2008).

Extracellular matrix components and proteases

ECM components and ECM proteolytic remodelling by proteases have also an important role in adipose tissue remodelling (Lijnen and Juhan-Vague 2002; Mariman and Wang 2010). Each adipocyte is surrounded by a basement membrane *in vivo* (Wang et al. 2008). Extracellular matrix

components, especially COL I, III, IV and VI are synthesized in adipose tissue at high levels (Maeda et al. 1997). ECM protein processing enzymes such as MMPs, ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs), PAI-1 and vaspin are important in maintaining the adipocyte ECM (Mariman and Wang 2010). During adipocyte formation, remodeling of ECM and increased basement membrane synthesis occur by secretion MMPs such as MMP-2 and MMP-9 (Alexander et al. 2001; Brown et al. 1997; Mannello 2006). In fact, the assembly of ECM is suggested to be the rate-limiting step in adipogenesis (Alexander et al. 2001). MMP activity regulators TIMPs (Visse and Nagase 2003), are released from connective tissue cells and macrophages in adipose tissue (Fain 2006).

Table I. The main endogenous factors released from adipose tissue

Class	Factor	Main cell source	Main function/effect	Involved in angiogenesis induction (+) or inhibition (-)	Elevated in obesity (+/-)	Main references
ENERGY AND LIPID METABOLISM REGULATORS	Leptin	adipocytes	major adipogenic and angiogenic hormone, endocrine effect in hypothalamus through sympathetic nervous system, inhibits feeding, increases energy use	+	+/-	(Bouloumie et al. 1998; Jequier 2002; Kim and Moustaid-Moussa 2000)
	Adiponectin	adipocytes	endocrine hormone, indicator of energy requirement, increases fatty acid and glucose catabolism, insulin sensitizing hormone	+	-	(Brakenhielm et al. 2004; Fain 2006; Gregoire 2001)
	Resistin	macrophages (humans)	hormone, link between obesity and diabetes, feedback signal to restrict adipocyte formation, overexpression of resistin leads to hyperglycemia	+	+	(Banerjee et al. 2004; Cao 2010; Patel et al. 2003; Rajala et al. 2004)
	Apelin	adipocytes	upregulated by insulin, role in type 2 diabetes, regulates cardiovascular functions	+	+	(Chudek and Wiecek 2006; Dray et al. 2010)
	Angiopoietin-like protein 4	adipocytes	LPL inhibitor, regulates endothelial cell survival and migration, target for PPAR γ	+/-	-	(Allhaud 2006; Cazes et al. 2006; Morisada et al. 2006)
	Visfatin	adipocytes	glucose lowering effect, increases in weight gain, dominantly expressed by visceral adipose tissue, increases cytokine production in monocytes	+	+	(Fukuhara et al. 2005; Kim SR et al. 2007; Moschen et al. 2007)
	Vaspin	adipocytes	potential insulin-sensitizer, increases in weight gain, visceral fat depot-specific secretory protein	+	+	(Hida et al. 2005; Kukla et al. 2011)
	Lipoprotein lipase (LPL)	adipocytes	uptake of lipids by adipocytes, gives rise to monoglycerides and fatty acids			(Wang et al. 2008; Allhaud 2006)
	Cholesterol ester transfer protein (CETP)	adipocytes	uptake of triglycerides	n/a	+	(Dusserre et al. 2000)
	Phospholipid transfer protein (PLTP)	adipocytes	mediate lipid uptake and transfer	n/a	+	(Dusserre et al. 2000)
	Apoprotein E (Apo E)	adipocytes	major regulator of lipid metabolism, mediates lipid turnover and transfer, modulates other lipid metabolism proteins	-	+	(Gao et al. 2007; Huang et al. 2007)

	Pigment-epithelium derived factor		contributes to insulin resistance in obesity, activates PPARY	-	+	(Chung et al. 2008; Crowe et al. 2009)
	endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS)	svf, endothelial cells	synthesizes nitric oxide, inhibits lipolysis, has a major role in adiponectin synthesis. iNOS is activated by inflammatory cytokines	+	+	(Elizalde et al. 2000; Engeli et al. 2004; Galvin et al. 2005; Koh et al. 2010; Murohara et al. 1998)
	nitric oxide (NO)	endothelial cells	inhibition of lipolysis	+	+	(Cooke and Losordo 2002; Engeli et al. 2004)
LIPIDS	Prostaglandins E ₂ and I ₂	svf, adipocytes	induce adipocyte differentiation	+	+/-	(Fain 2006; Ailhaud 2006)
	Monobutyrin	adipocytes, preadipocytes	proangiogenic lipid	+	+	(Dobson et al. 1990; Wilkison et al. 1991)
	Free fatty acids (Non-esterified fatty acids)	adipocytes	stimulate lipid uptake and metabolism, induce adipocyte differentiation, cause insulin resistance and inflammation	-	+	(Ailhaud 2000; Boden 2008; Karpe et al. 2011; Kim and Moustaid-Moussa 2000; Mohamed-Ali et al. 1998)
	Steroid hormones (estrogens, androgens, cortisol)	adipocytes	reduce fertility, change local activity of sex hormones	+	+/-	(Mayes and Watson 2004; Mohamed-Ali et al. 1998)
	Retinol binding protein -4	adipocytes	retinol carrier, induces insulin resistance, increases blood glucose level	+	+	(Galic et al. 2010; Toyama et al. 2011; Yang et al. 2005)
CYTOKINES AND CHEMOKINES	VEGF(-A)	svf, adipocytes	induces endothelial cell proliferation and migration and vascular permeability	+	+	(Fain 2006; Wang et al. 2008; Christiaens and Lijnen 2010; Otsrock et al. 2007; Carmeliet and Jain 2011; Silha et al. 2005)
	VEGF-C	svf, adipocytes	activates endothelial tip cells	+	+	(Christiaens and Lijnen 2010; Silha et al. 2005)
	VEGF-D	svf, adipocytes	involved in lymphangiogenesis	+	+	(Stacker et al. 2001; Silha et al. 2005)
	PlGF	svf, adipocytes	increases revascularization in ischemia, wounds and tumors	+	n/a	(Carmeliet and Jain 2011)
	HGF	svf	induces adipogenesis	+	+	(Cao 2010; Rehman et al. 2004)

	FGF-1	adipocytes, preadipocytes	induces adipogenesis, regulates PPAR γ	+	+	(Jonker et al. 2012; Mejhert et al. 2010)
	bFGF	adipocytes, preadipocytes	proliferatory cytokine, induces endothelial cell migration, proliferation and differentiation, upregulates e.g. HGF and IL-6 and induces adipogenesis	+	+	(Carmeliet and Jain 2011; Rehman et al. 2004; Kawaguchi et al. 1998; Mejhert et al. 2010)
	PDGF-BB	macrophages, endothelial cells	contributes to angiogenesis, has an important role in the maturation of vasculature	+	n/a	(Hellstrom et al. 1999; Mornex et al. 1986; Shimokado et al. 1985; Wang et al. 2012)
	TGF- β	macrophages, other svf cells	multifunctional cytokine, at low levels upregulates angiogenic factors, at high levels contributes to tubule maturation, tissue remodeling and wound repair, inhibits adipocyte differentiation	+/-	-	(Christiaens and Lijnen 2010; Bourlier et al. 2008; Holderfied and Hughes 2008; Pardali et al. 2010)
	IL-6	svf cells (T-cells and macrophages), part from adipocytes	regulates production of other cytokines (activates IL-1ra and IL-10, inhibits TNF- α and IL-1), increases acute-phase protein (CRP, haptoglobin, amyloid protein) secretion in liver, enhances glucose uptake in muscle, decreases lipogenesis, key role in the development of coronary heart disease, enhances tissue repair	+	+	(Butler et al. 2010; Fain 2006; Lazar 2005)
	IL-1 β	svf	contributes to development of insulin resistance	+	+	(Fain 2006; Koenen et al. 2011)
	IL-8	macrophages, T-cells other svf	important in obesity related low-grade inflammation, induces adipocyte survival by stimulating angiogenesis	+	+	(Shoshani et al. 2005)
	IL-10	svf	feedback inhibitor of the effects of proinflammatory cytokines, increased in obesity	-	+	(Fain 2006)
	IL-1Ra	svf	antagonist of IL-1 α and IL-1 β , contributes to weight gain and inflammation	n/a	+	(Fain et al. 2006; Juge-Aubry et al. 2003)
	IL-18	svf	contributes to insulin resistance and both type I and type II diabetes	+	+	(Amin et al. 2010; Escobar-Morreale et al. 2004; Leick et al. 2007)
	GM-CSF	svf (T-cells)	proinflammatory cytokine, reduces food intake, contributes to macrophage infiltration to adipose tissue	+	+	(Cao 2010; Kim et al. 2008; Yang et al. 2010)
	TNF- α	activated macrophages main source	stimulation of proinflammatory cytokine secretion of monocytes/macrophages, role in insulin resistance, TNF activation can begin and maintain inflammation	+/-	+	(Chudek and Wiecek 2006; Fain 2006; Fain et al. 2004)
	TNF- β (lymphotoxin- α)	preadipocytes, adipocytes	induces angiogenesis	+	n/a	(Cao 2010)

	Stromal derived factor 1	svf, endothelial cells	contributes to angiogenesis	+	n/a	(Cao 2010)
	Ang-1	adipocytes, svf	glycoprotein that contributes to vessel maturation and stability, constitutively activates Tie-2, induces recruitment of pericytes	+	-	(Christiaens and Lijnen 2010; Dallabrida et al. 2003; Davis et al. 1996; Paul et al. 2012)
	Ang-2	endothelial (tip) cells	counteracts with Ang-1, induces angiogenesis	+	+	(Cao 2010; Lijnen 2008; Silha et al. 2005;)
	Angiogenin	svf, adipocytes	induces angiogenesis by activating endothelial cells and smooth muscle cells, also known as ribonuclease 5 with enzymatic activity	+	+	(Gao and Xu 2008)
	Macrophage migration inhibitory factor (MIF)	svf, adipocytes	recruitment of macrophages to adipose tissue	+	+	(Dandona et al. 2004; Fain et al. 2006; Kim HR et al. 2007)
	MCP-1	svf cells	recruitment of monocytes/macrophages into adipose tissue	+	+	(Ailhaud 2006; Fain 2006; Niu et al. 2008; Sartipy and Loskutoff 2003)
	Osteonectin/SPARC	adipocytes	elevates plasma PAI-1 in obesity, binds to VEGF, inhibits bFGF and inhibits endothelial cell proliferation	-	+	(Gregoire 2001; Tartare-Deckert et al. 2001)
	RANTES (CCL5)	svf (T-cells)	interacts with GPCRs (G-protein-coupled receptors) and GAGs (glycosaminoglycans), contributes to angiogenesis	+	-	(Skopkova et al. 2007; Suffee et al. 2011)
	IGF-1	preadipocytes, adipocytes	stimulates adipogenesis, activates SREBP1c	+/-	+/-	(Bucky and Percec 2008; Nam et al. 1997; Shigematsu et al. 1999; Wabitsch et al. 2000)
	IGFBP-3	preadipocytes, adipocytes	binds IGF-1 and IGF-2, impairs PPAR γ induced adipogenesis, increases PAI-1, upregulates angiogenic genes	+	-	(Chan et al. 2009; Granata et al. 2007; Nam et al. 1997; Wabitsch et al. 2000)
	Nerve growth factor (NGF)	svf, adipocytes	modulation of innervation, inflammatory response protein	+	+/-	(Fain et al. 2006; Peeraully et al. 2004)
	HIF-1 and HIF-2	svf cells	increases expression of angiogenic factors such as VEGF, downregulates endogenous angiogenesis inhibitors	+	+	(Cao 2010; He et al. 2011; Hosogai et al. 2007)
ECM COMPONENTS	Tsp-1, Tsp-2	adipocytes, platelets	cell-matrix and cell-cell interactions, inhibits angiogenesis	-	-	(Alvarez-Llamas et al. 2007; Cao 2010; Christiaens and

								Lijnen 2010)
	Tissue factor (TF)	endothelial cells		main coagulation activator, highly abundant in angiogenic vessels, promotes insulin resistance and obesity	+		+	(Bussolino et al. 1997; Zhang and Lawrence 2011)
	Cathepsin B	svf		regulates pro- and anti-angiogenic factors, possible angiogenic switch of endothelial cells, modulates ECM	+/-		+	(Alvarez-Llamas et al. 2007; Im et al. 2005)
	Cathepsin S	svf		link between obesity and atherosclerosis	+		+	(Fain et al. 2006; Naour et al. 2010)
	MMP-2, -9	svf		ECM degradation and remodeling	+		-	(Bouloumie et al. 2001; Cao 2010)
	TIMP-1	svf (macrophages)		inhibits MMP activity	-		+	(Cao 2010; Maquoi et al. 2002)
	TIMP-2	svf (macrophages)		inhibits MMP activity	-		-	(Maquoi et al. 2002)
	PAI-1	svf, adipocytes		procoagulative agent and fibrinolysis inhibitor, increased plasma levels in obese, systemic effect in atherothrombosis, increased PAI-1 stimulates release of PDGF	-		+	(Ailhaud 2006; Alessi and Juhan-Vague 2004; Alessi and Juhan-Vague 2006; Chudek and Wiecek 2006)
RENIN-ANGIOTENSIN SYSTEM	Angiotensinogen	adipocytes		peptide hormone, part of the renin-angiotensin system, that causes blood vessels to constrict, and increases blood pressure recruits new adipocytes via adipogenesis, elevates blood pressure in obese	-		+	(Ailhaud et al. 2002; Celerier et al. 2002; Yvan-Charvet and Quignard-Boulange 2011)
	Angiotensin II			vasoactive component of the renin-angiotensin system, contributes to increased blood pressure, increases nitric oxide production	+		+	(Engeli et al. 2004; Goossens et al. 2003; Kim and Moustaid-Moussa 2000; Tamarat et al. 2002; Yvan-Charvet and Quignard-Boulange 2011)
COMPLEMENT SYSTEM COMPONENTS	Adipsin (complement factor D)	adipocytes, macrophages		used for synthesis of Acylation-stimulating protein (ASP) together with complement factor B and C3, all produced by adipose tissue. Adipsin is a rate limiting factor in the complement activation	+		-	(Ailhaud 2006; Rosen et al. 1989)
	Acylation stimulating protein (ASP)	adipocytes		stimulates triglyceride synthesis	n/a		+	(Ailhaud 2006; Xia et al. 2002)

Excess adipogenesis and cytokine release

Obesity is characterized by an increase in adipocyte size and number, changes in the levels of adipocytokine secretion, and recruitment of macrophages that release the pro-inflammatory cytokines. Excess adipogenesis leads to chronic inflammation and obesity that is related to various diseases such as type 2 diabetes, hypertension, dyslipidemia and cardiovascular disease. Obesity is also related to diseases such as certain cancers and depression. Obesity is defined as a body mass index (BMI) of 30kg/m^2 or more. (Daquinag et al. 2011) Obesity is a major health problem in the world. According to World Health Organization (WHO), 1.6 billion adults are overweight, and at least 400 million are obese. (Wang et al. 2008; WHO, 2011) Highly metabolically active visceral adipose tissue is known to be in a predominant role in the development of obesity related diseases (Fain et al. 2004; Fain 2006), and visceral fat accumulation is therefore a risk factor for cardiovascular disease, cerebrovascular disease, hypertension and type 2 diabetes (Carr et al. 2004; Chudek and Wiecek 2006).

The excess intake of lipids induces adipocytes to produce more pro-inflammatory cytokines and chemoattractant molecules. Indeed, the chronic inflammation is characterized by a strong increase in the release of inflammation markers such as TNF α , IL-1 β , IL-6, IL-8, IL-10, MCP-1, PAI-1, colony stimulating factor (CSF) and iNOS. (Fain et al. 2004; Feghali and Wright 1997; Neels and Olefsky 2006; Wang et al. 2008) Several angiogenic factors, e.g. VEGF-C, VEGF-D, Ang-2, angiogenin and endostatin are also significantly elevated in overweight subjects (Christiaens and Lijnen 2010). In obesity, in addition to the macrophage number, the number of neutrophils and T lymphocytes are also increased (Elgazar-Carmon et al. 2008; Ohmura et al. 2010; Weisberg et al. 2003). The induction of inflammatory changes is due to endoplasmic reticulum stress, hypoxia and oxidative stress (Furukawa et al. 2004; Hosogai et al. 2007; Ozcan et al. 2004). The tissue hypoxia induces apoptosis and necrosis of adipocytes in obese (Strissel et al. 2007) which further induce macrophage recruitment into tissue and inhibit ASC differentiation into adipocytes by suppressing e.g. PPAR γ activity, but favor ASC differentiation into endothelial cells (Ye and Gimble 2011). Local inflammation induces angiogenesis through induction of angiogenic factors and therefore improves the tissue blood supply (Pang et al. 2008; Ye and Gimble 2011). On the other hand, to maintain the tissue volume, preadipocytes and adipose stem cells pursue to replace the dead cells by differentiating into adipocytes. Therefore, there is competition between adipogenesis and endothelial cell differentiation of ASC. Meanwhile, the remaining adipocytes maintain the tissue volume by storing triglycerides and increasing in size. (Ye and Gimble 2011)

Adipose tissue macrophages

Lean adipose tissue has resident macrophages that express e.g. IL-10 (Lumeng et al. 2007). These cells have increased capacity to contribute to tissue repair and angiogenesis (Galic et al. 2010). Macrophages secrete several angiogenic factors and cytokines e.g. VEGF-A, IL-6, IL-8, TNF and chemokines (Hotamisligil 2006; Wang et al. 2008), resistin (Patel et al. 2003) and are a main source of PDGF-B in tissue (Mornex et al. 1986; Shimokado et al. 1985).

Macrophages have an essential role in in adipose tissue remodeling, obesity and metabolic syndrome (Suganami and Ogawa 2010). During tissue stress such as adipose tissue expansion during obesity or tissue response to implantation of grafts, cytokines and endothelial cells recruit monocytes from bone marrow to adipose tissue, that then differentiate into macrophages (Shoelson et al. 2006). As adipocyte necrosis is 3-times higher with obese than lean people, infiltrating macrophages are suspected to be recruited to tissue to destroy the necrotic adipocytes (Cinti 2006; Wang et al. 2008). Macrophages undergo phenotypic transformation in obese adipose tissue from M2 anti-inflammatory phenotype into activated M1 proinflammatory phenotype macrophages (Hibino et al. 2011; Lumeng et al. 2007; Suganami and Ogawa 2010; Weisberg et al. 2003; Wellen and Hotamisligil 2003) that have increased expression of pro-inflammatory cytokines such as interleukins, TNF α and iNOS (Lumeng et al. 2007). Infiltrated macrophages are mainly responsible for the chronic inflammation and increased production of cytokines in obesity (Fain 2006; Odegaard and Chawla 2008; Weisberg et al. 2003).

Angiogenic macrophages are activated through hypoxia-dependent VEGF-A signaling system (Cho et al. 2007). MCP-1 and chemokine (C-C motif) receptor 2 (CCR2) induce the macrophage recruitment into adipose tissue (Ito et al. 2008; Kanda et al. 2006). Macrophages are localized to dead adipocytes where they fuse to form multinucleate giant cells, a sign of chronic inflammation, in order to scavenge the dead cells and lipids (Cinti et al. 2005). Macrophages are known to stimulate angiogenesis in obese adipose tissue (Pang et al. 2008) and suppress the formation of mature adipocytes and to increase the inflammatory factor production in preadipocytes (Lacasa et al. 2007; Suganami and Ogawa 2010). They therefore may also limit adipose tissue expansion (Lacasa et al. 2007).

A schematic drawing of adipose tissue expansion and the development of obesity is seen in Figure 3.

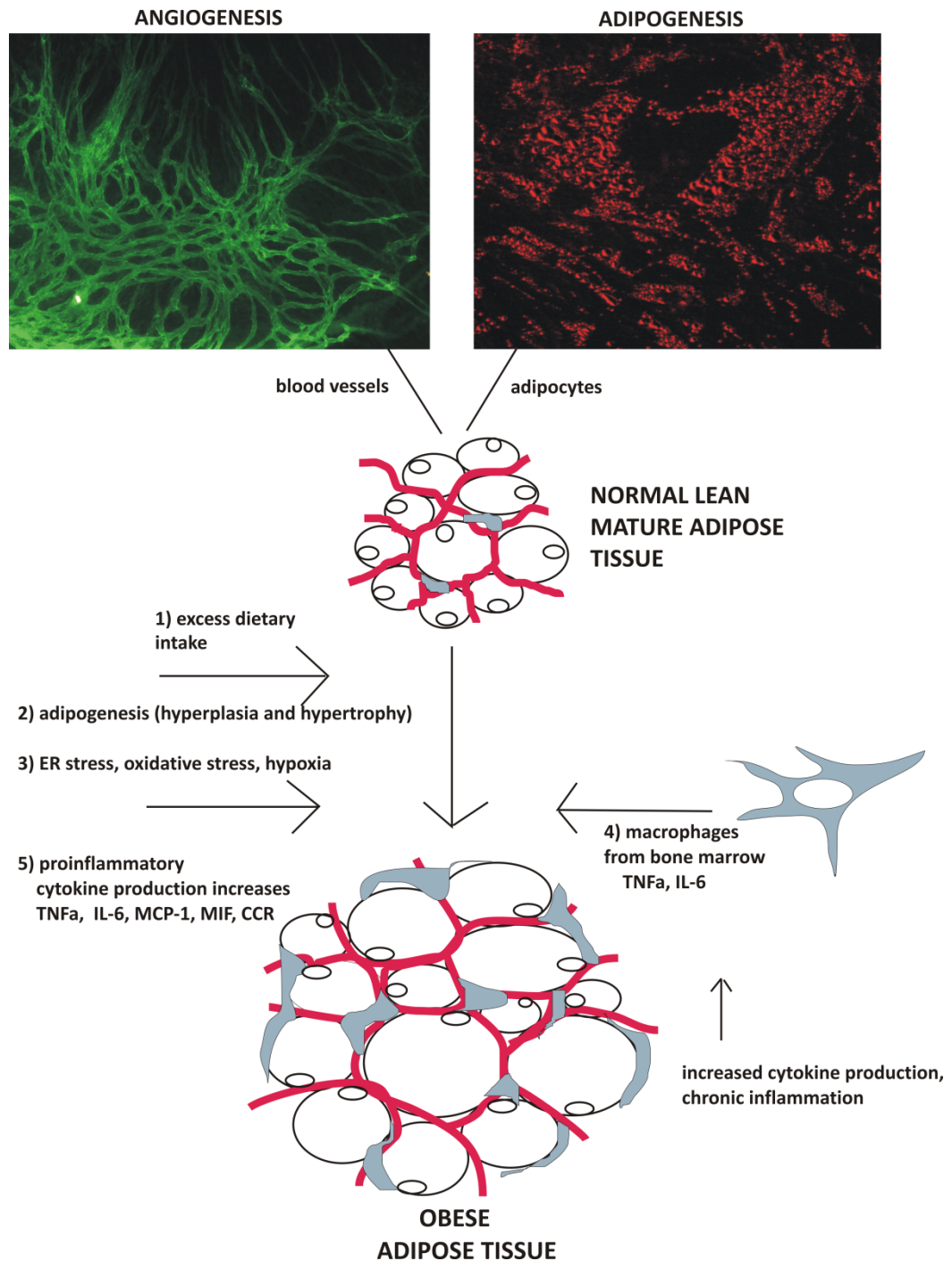


Figure 3. Adipose tissue expansion and development of obesity. During adipose tissue formation, adipogenesis and angiogenesis occur simultaneously, leading to formation of normal lean adipose tissue. Due to e.g. excess dietary intake, abnormal adipose tissue expansion begins. This leads to endoplasmic reticulum (ER) stress, oxidative stress, hypoxia and adipocyte death, that leads to increased proinflammatory cytokine production. These phenomena induce macrophage migration into adipose tissue and transformation into proinflammatory phenotype, which leads to further increased cytokine production and chronic inflammatory stage.

Soft tissue engineering

Clinical need for soft tissue engineering

Soft tissue consists of structures supporting the body such as muscles, connective tissue, adipose tissue and blood vessels. Soft tissue engineering seeks to fabricate replacement parts for soft tissue defects (burns, scars and chronic wounds), surgical resections, and congenital malformations. Particularly congenital or trauma related facial defects are a challenge to soft tissue surgery. Cosmetic use, such as rejuvenation (filling of facial wrinkles) and breast augmentation are also important applications of reconstituted soft tissue. (Choi et al. 2010; Masuda et al. 2004a; Patrick 2001; Schaffler and Buchler 2007)

Soft tissue engineering is a prominent research area, and soft tissue defects and chronic wounds are a medical and economic challenge to the health care system with the demand for the treatment of soft tissue defects increasing every year (Ferreira et al. 2006; Levin and Condit 1996). The major causes for chronic wounds are diabetic neuropathy and obstruction of main blood vessels (Ferreira et al. 2006). The foot ulcers, pressure ulcers and active venous ulcers affect altogether 650 000 patients only in United Kingdom, with a cost of even £3 billion a year (Bennett et al. 2004; Simon et al. 2004). In the United States, there are 5 million wound patients that generate annual costs in excess of \$20 billion for health care and the numbers are growing 10% annually (Levin and Condit 1996). Moreover, 13.8 million cosmetic plastic surgery procedures (both surgical and minimally-invasive) and 5.5 million reconstructive plastic surgery procedures (of which 4.2. million are tumor removals) were performed in the United States in 2011. According to ASPS, cosmetic procedures have experienced growth of nearly 600 percent in the last decade. (ASPS, 2011) Soft tissue defects often also impair function and affect the patients emotionally (Patrick 2001). For all of these reasons, there is an urgent clinical need for adipose tissue substitutes.

Despite the progress in soft tissue engineering during recent years, tissue-engineered adipose tissue is still a great challenge, and the current applications have shown only modest success e.g. some vascularization but hardly any adipose tissue formation (Neels et al. 2004). Solely the revascularization of ischemic tissues would benefit millions of people, but therapeutic angiogenesis and the optimal application for the reconstruction of soft tissue remain an unmet clinical need (Carmeliet and Jain 2011; Torio-Padron et al. 2007; Vermette et al. 2007).

Key requirements for soft tissue replacement

Biocompatibility, bioactivity and sustainability are the main goals for the optimal soft tissue implant. The ideal soft tissue replacement should first rapidly induce functional vascularization and induce adipogenesis with permanent effect. (Choi et al. 2010) Moreover, the regenerated adipose tissue should mimic endogenous tissue both in function and in structure (Hausman et al. 2001). Therefore, the metabolic and endocrine activity of adipose tissue should also be taken into account (Giorgino et al. 2005). The degradation properties of the selected biomaterial are also important to consider, as especially natural biomaterials are typically easily degraded by tissue proteases (Lavik and Langer 2004). Moreover, the implanted construct should not induce immunorejection in host (Levenberg and Langer 2004). Minimal donor-site morbidity is also a prerequisite (Choi et al. 2010).

Present clinical methods for soft tissue repair

Current therapeutic procedures for soft tissue replacement include filling the defects with synthetic or natural biomaterials, often with injectable systems. Also autologous fat grafts as well as stem cell therapy, where stem cells are usually incorporated into biomaterial, are common. (Badylak 2002; Choi et al. 2010; Gomillion and Burg 2006; Hanson et al. 2011; Hemmrich et al. 2005; Patrick 2001)

Clinically, the mostly used soft tissue augmentation procedures are breast reconstructions (Patrick 2001). Current surgical breast tissue therapies include autologous vascularized flaps or synthetic nonvascularized approaches. Autologous vascularized flaps consist of adipose tissue which is transplanted with its blood supply (Tachi and Yamada 2005). These flaps may also contain muscle and fascia tissue (Butterwick et al. 2007). However, with the current methods, significant donor-site morbidity occurs (Tachi and Yamada 2005, Wu et al. 2008) and recipient-site morbidity is also common (Wu et al. 2008). Nonvascular alternatives for breast reconstruction include silicone implants, known to represent difficult long-term complications such implant rupture that leads to severe foreign body reactions (Beahm and Walton, 2009).

ASC, known to secrete a wide number of inductive factors and have an ability to induce adipogenesis (Zuk et al. 2001), angiogenesis (Rehman et al. 2004) and wound healing (Altman et al. 2009), have currently become the most popular choice for adipose tissue engineering (Choi et al. 2010). Adipose tissue is an attractive and advanced source for soft tissue repair as it is abundant and easy to obtain with minimal invasive methods (Christiaens and Lijnen 2010; Strem and Hedrick 2005; Traktuev et al. 2008). Furthermore, the methods for adipose stromal cell isolation and differentiation are well described in the literature (Bunnell et al. 2008;

Gimble and Guilak 2003; Zuk et al. 2001). As natural components of adipose tissue, ASC are also the most natural source for adipose tissue engineering, and compared to e.g. bone marrow stem cells, ASC have advantages such as low donor-site morbidity (Miranville et al. 2004).

De novo soft tissue formation

Inductive adipogenesis/angiogenesis (*de novo* adipogenesis/angiogenesis) is defined as the use of cellular tissue engineering devices, where stimulus is applied *in vivo* as inductive growth factors. The inductive microenvironment enhances migration of adipose stromal cells in the surrounding tissue and induces the cells to differentiate into mature adipocytes. (Kim et al. 2012)

The adequate induction of angiogenesis is a prerequisite for successful adipose tissue reconstruction (Kim et al. 2012; Rivron et al. 2008; Verseijden et al. 2009). Neovascularization is required for the developing adipose tissue for providing nutrients and essential differentiation factors and therefore to avoid necrosis and scar formation (cao 2010). Acellular soft tissue substitute that would induce its own replacement, would be advantageous to soft tissue engineering (Kelly et al. 2006; Marra et al. 2008; Neels et al. 2004). Inductive adipogenesis would not require stem cell harvest from the patient and acellular alternative would likely have less immune reactions and therefore the alloplastic use of the implant could be possible. (Kelly et al. 2006; Kimura et al. 2002; Masuda et al. 2004a; Masuda et al. 2004b; Patrick et al. 2002; Tabata et al. 2000; Vashi et al. 2006; Yuksel et al. 2000a)

The neovasculature in tissue engineered vascular grafts has been reported to be derived from the host cells, via vascular remodeling, instead of the implanted cells (Neels et al. 2004; Roh et al. 2010). The inductive key molecules are therefore suggested to be sufficient for the induction of vascular development (Roh et al. 2010). Indeed, several studies have been performed to provide a suitable microenvironment for blood vessel and adipose tissue induction. This has been tried by incorporating growth factors or extracellular matrix proteins into biomaterials (Kawaguchi et al. 1998; Kelly et al. 2006; Masuda et al. 2004a; Neels et al. 2004; Nillesen et al. 2007; Yuksel et al. 2000b; Yuksel et al. 2000c). Induction of adipogenesis has been studied with gelatin based microspheres with incorporation of bFGF, insulin and IGF-1 (Tabata et al. 2004). Matrigel with bFGF has also been used (Marra et al. 2008). Epidermal growth factor (EGF), TGF- β and PDGF have also been shown to induce adipogenesis (Gomillion and Burg 2006). The controlled release of multiple angiogenic factors is therefore beneficial for tissue regeneration. (Neels et al. 2004; Rehman et al. 2004; Semenza 2003) However, finding the optimal cocktail of molecules and to deliver them in the most optimal way for inductive angiogenesis and adipogenesis is still difficult, especially in long-term (Patrick 2001).

Biomaterials for soft tissue engineering

To regenerate functional tissue, cells and inductive factors are most often cultured with an artificial extra cellular matrix, a biomaterial scaffold that will aid in the cell attachment, proliferation and differentiation. Scaffolds are divided in to two different types, synthetic polymer scaffolds and natural scaffolds. The prerequisites for scaffolds used in vascular engineering or soft tissue engineering are biocompatibility, biodegradability, and structure that allows cell interaction (Choi et al. 2010). The structure (rigidity and pore size) of the scaffold plays a significant role in differentiation induction. (Choi et al. 2010; Stacey et al. 2009)

Synthetic polymers

Synthetic polymers are widely used in adipose tissue engineering, as their mechanical and chemical properties and degradation rate are easily modified (Lavik and Langer 2004). The most commonly used synthetic polymers are biodegradable polymers poly(lactic acid) (PLA), poly(glycolic acid) (PGA) or their co-polymer poly(lactic-co-glycolic acid) (PLGA) (Cho et al. 2005; Choi et al. 2010). Silicone, a non-degradable synthetic material, is commonly commercially used for breast tissue augmentations (Patrick 2001). However, synthetic materials have drawbacks such as unwanted allergic or foreign body reactions, resorption of transplants or poor adipogenesis and angiogenesis induction (Gomillion and Burg 2006).

Natural polymers

Natural polymers are components of extracellular matrix or materials generated by biological systems (Lavik and Langer 2004). Natural polymers provide rapid interaction with host cells, induce host cell migration to graft and deposition of additional ECM (Laschke et al. 2005). Overall, they have advantages such as good biocompatibility and biological properties that match to the host tissue (Choi et al. 2010). Naturally occurring hydrogels are usually non-toxic and less severe foreign body reactions occur, but they also have certain limitations in their use. Natural biomaterials are often structurally rigid, degrade easily in tissue and have poor mechanical properties (Hemrich et al. 2008; Ko et al. 2007). Moreover, in some cases, naturally derived materials may trigger severe immune responses in patients (Schmidt and Baier 2000).

Several ECM components such as COL I, fibronectin, gelatin, hyaluronan, fibrin, decellularized human placenta, amnion membrane, adipose derived hydrogels, Matrigel, silk, small intestinal submucosa, acellular dermis and amniotic membrane have been used for adipose tissue

engineering (Patrick 2001; Laschke et al. 2005; Choi et al. 2010). Probably the most widely used natural material is COL I. It is homologous between species and a major structural component in the body (Brown et al. 2000) and therefore biocompatible (Vashi et al. 2006). Matrigel, a collagen-based growth factor rich gel matrix from mouse sarcoma, promotes adipogenesis and angiogenesis (Cronin et al. 2007). However, the use of Matrigel is problematic as it is a tumor-derived and xenogenic material and moreover, not thought to be very inductive material, rather only conductive (Chiu et al. 2011). Therefore, other potential adipogenic and angiogenic materials are needed and searched for clinical use (Kelly et al. 2006). Recently, autologous and allogenic injectable hydrogel fillers, especially hyaluronic acid-based materials, have become an especially attractive option for soft tissue engineering due to the minimal invasive procedures needed (Bucky and Percec 2008; Rohrich et al. 2007).

Hyaluronic acid

Hyaluronic acid (HA) is a glycosaminoglycan that consists of a basic unit of two sugars, glucuronic acid and *N*-acetylglucosamine, that are polymerized into large macromolecules (Badylak 2002). HA is a common natural component of ECM among many organisms and present in connective tissues of skin, cartilage, bone and synovial fluid. HA is structurally conserved regardless of the source and therefore it is considered nonallergenic. (Rohrich et al. 2007; Badylak 2002). It is unique as it is natively present in the intracellular matrix of the dermis (Laurent 1987). HA takes part in binding growth factors and cytokines (Badylak 2002). HA is known to modulate inflammation and tissue reparation (Hanson et al. 2011) and to promote angiogenesis (Hemmerich et al. 2008; Ventura et al. 2007) and is therefore considered a very potential material for soft tissue engineering. (Hanson et al. 2011) HA has been used for inducing bone and cartilage (Yuksel et al. 2000), as dermal filler (Rohrich et al. 2007), in vocal fold regeneration (Sahiner et al. 2008) and in soft tissue induction (Patrick 2001; Wu et al. 2007). The degradation products of HA are thought to be able to also modulate wound healing (Rohrich et al. 2007).

Natural HA is rapidly degraded in the liver to carbon dioxide and water (DeVore et al. 1994) and therefore it lacks potential utility for adipose tissue engineering (Hemmerich et al. 2008). Therefore, the commercial HAs currently used are mainly crosslinked. Restylane® (Q-Med) and Juvederm® (Allergan Inc.) are partially cross-linked HA derivatives obtained with bacterial fermentation. This crosslinking results in viscoelastic transparent gel (Rohrich et al. 2007). Hylaform™ (Genzyme Corporation), another commercial HA, is derived from avian proteins (Rohrich et al. 2007). Several FDA approved injectable hyaluronan hydrogel materials are already in

clinical use and the adverse reactions of HA fillers have been minimal and mainly injection-related (Friedman et al. 2002; Rohrich et al. 2007).

The popularity of HA has increased dramatically since 2004, when approximately 45 000 treatments were administered by ASPS member surgeons as a filler for cutaneous lines and wrinkles. By the year 2011 the number of these minimally invasive procedures with HA increased almost 30-fold, being more than 1.3 million procedures in a year. According to ASPS, soft tissue fillers like hyaluronic acid (Restylane®, Juvederm Ultra®) and fat injections experienced some of the largest growth in the minimally-invasive market during 2011 (increase in 2011 vs. 2010 for HA fillers 9%, fat injections 19%), whereas collagen injections had decreased at 2011 55% from 2010. Moreover, HA fillers are the far most popular treatment for soft tissue filling. (ASPS, 2011)

In vitro vascular analogues

Due to complex interactions during angiogenesis, the evaluation of factors that affect angiogenesis would optimally be studied *in vivo*. The most commonly used *in vivo* angiogenesis assays include chick chorioallantoic membrane (CAM) assay, Matrigel plug assay, zebrafish embryo system, corneal micropocket assay, rat/mouse hindlimb ischemia model, rat aortic ring assay, intradermal angiogenesis assays and *Xenopus* tadpole assay. (Auerbach et al. 2003). These *in vivo* assays are used to measure new blood vessel formation by pro- and anti-angiogenic compounds (Akhtar et al. 2002; Auerbach et al. 2003; Ishikane et al. 2008; Norrby 2006; Ziche and Morbidelli 2009), to study tumor angiogenesis (Auerbach et al. 2003; Norrby 2006) or embryonic and organogenic angiogenesis (Norrby 2006). Although animal assays do not reflect the effect in human body and disease as regards both efficacy and relevance and are difficult to interpret (Ishikane et al. 2008; Norrby 2006), they are widely used as they are considered to provide more information on complex cellular and molecular interactions compared to *in vitro* models (Norrby 2006).

In vitro angiogenesis assays are regarded as useful tools for screening compounds, their toxicity and effective concentrations in the initial stage of safety testing of chemicals, but due to complex interactions during angiogenesis, they often need to be followed by *in vivo* studies (Auerbach et al. 2003) and do not allow the complete replacement, but reduction of animal testing (Balls et al. 1995). An ideal *in vitro* assay should contain all the relevant steps of human angiogenesis including vessel maturation (Vailhe et al. 2001), it should measure both stimulation and inhibition (Bishop et al. 1999; Norrby 2006), provide easy quantitative measurement of the tubule formation (Norrby 2006), and cost-effective, fast, reproducible and reliable routine assay (Jain et al. 1997; Vailhe et al. 2001). The first capillary-like structures developed *in vitro* were endothelial cells

grown on plastic (Folkman and Haudenschild 1980) and on collagen gels (Montesano et al. 1983). Capillary-like structures have later been developed by co-culturing endothelial cells with dermal fibroblasts and keratinocytes (Black et al. 1998) and with mouse myoblasts and embryonic fibroblasts (Levenberg et al. 2005). In addition to microvascular EC (Folkman and Haudenschild, 1980) and HUVEC (Black et al. 1998; Levenberg et al. 2005), endothelial progenitor cells have also been used (Shepherd et al. 2006).

The current *in vitro* assays can be divided into two categories, two-dimensional and three-dimensional assays. In two dimensional assays, endothelial cells form tubules in the presence of e.g. collagen I, III or IV/V, Matrigel, fibronectin or gelatin. The two-dimensional assays do not reflect all the steps of angiogenesis, but are considered convenient for screening of angiogenic molecules. In three-dimensional assays, endothelial cells form tubular structures in gelified substrates (e.g. collagen, Matrigel, fibrin). (Vailhe et al. 2001)

The disadvantages of the current *in vitro* assays are the lack of metabolism and rare use of normal human primary cell based assays (Auerbach et al. 2003). Most of the current angiogenesis assays have also dramatic variability or the methods lack quantification and clinical relevance (Auerbach et al. 2000). Most cell culture assays (e.g. tubule formation assays in collagen or Matrigel, cell motility assays, trans-membrane assays or cell proliferation assays) only reflect one single step of angiogenesis and critical cell-cell or cell-matrix interactions are limited (Auerbach et al. 2003). Although tubule formation *in vitro* does not cover the whole angiogenesis process, it effectively mimics the key steps, i.e. migration and differentiation of endothelial cells (Donovan et al. 2001). Co-culture assays that have been used in several studies (Bishop et al. 1999; Donovan et al. 2001; Friis et al. 2005; Friis et al. 2003; Merfeld-Clauss et al. 2010; Montesano et al. 1993; Nicosia and Ottinetti 1990) provide a useful method for studying angiogenesis in controlled conditions *in vitro*, with the advantage of being able to replicate some tissue-derived signaling (Rubina et al. 2009).

Validation of in vitro assays

One of the most critical technical problems is the accurate interpretation of highly variable results from different *in vitro* (angiogenesis) assays (Folkman et al. 2001; Jain et al. 1997). Methods vary dramatically in what they measure, are limited in the relevance to human and often lack quantitative measurements (Auerbach et al. 2000). An important goal would be to standardize the current *in vitro* assays and to develop assays that reflect the *in vivo* situation (Vailhe et al. 2001). A valid *in vitro* assay should give similar results in different laboratories, and therefore standardization between laboratories is essential (Piersma 2004).

Good Laboratory Practice (GLP) is a quality system under which the non-clinical health and environmental safety studies are planned, performed, monitored, recorded, archived and reported. The principles of GLP should be applied to the non-clinical safety testing of test items including pharmaceuticals, pesticides, cosmetics, veterinary drugs, food additives and industrial chemicals. (OECD, 1998) The GLP system is traditionally designed for safety studies, but the principles of GLP can be applied to all *in vitro* studies (OECD, 2004), including efficacy studies. The important requirements of GLP are trained and qualified personnel; adequate laboratory facilities, equipment and materials; accurate recordings of the qualifications, training and experience and establishment of Standard Operating Procedures for the laboratory (Balls et al. 1995; Gimble et al. 2011). For each study, a study plan must be written and followed, and changes need to be documented with amendments to the original protocol. The responsibilities of the personnel for each study must be addressed. Importantly, the origin of the biological system, such as cells, must be well defined and characterized and the stability must be assured (Balls et al. 1995).

The establishment of a routine test method contains three stages, method development, method optimization and method validation (FICAM 2010). The test development phase defines the scientific base of the method as well as the need of the method and method optimization defines the detailed protocol for the method (Balls et al. 1995). The method validation is a process, where the reliability and relevance of a test system is established (Balls 1990) and it is generally described in and guided by the OECD regulatory Guidance Document No 34 (OECD 2005). For *in vitro* method validation, the overall responsibility of the final validation process is with an official authority, which in European Union (EU) is The European Reference Laboratory on Alternatives to Animal Testing (EURL ECVAM) that was founded by the European Commission to coordinate the validation of alternative methods in the European Union (Curren et al. 1995). EURL ECVAM action is directed by EU Directive 2010/63/EU (EC 2010). The EURL ECVAM validation process consists of several stages: test development, intra-laboratory validation (called as pre-validation in the process), inter-laboratory validation, independent assessment and progression towards regulatory acceptance. During intra-laboratory validation the protocol is standardized. (Balls et al. 1995) The intra-laboratory validation phase defines the robustness and reproducibility of the test method and how well it predicts the specific *in vivo* end-points (Curren et al. 1995). The main purpose of the validation is to conduct an inter-laboratory blind trial of the methods. The formal validation study contains a preliminary phase, transfer phase and a definitive validation phase. After validation, the results of the blinded study are considered by independent assessment panels and finally the validated method will progress towards regulatory acceptance for OECD guidelines. (Balls et al. 1995) Only formally inter-laboratory validated methods can be accepted by the regulatory authorities.

Importantly, the validation of the *in vitro* test system includes the comparison of the responses obtained in the specific *in vitro* test with known *in vivo* responses. The extrapolation of the *in vitro* effects is challenging and mathematical modeling can be used to predict the results. (Piersma 2004)

Aims of the study

The specific aims of the present study were:

1. To extract adipogenic and angiogenic factors from mature adipose tissue and to characterize the extract (I)
2. To study the angiogenic and adipogenic properties of the adipose tissue extract in respective *in vitro* assays (I)
3. To study the potential of the adipose tissue extract to induce capillary proliferation and soft tissue formation *in vivo*, especially in long-term (II)
4. To study the biocompatibility of the adipose tissue extract *in vivo* (II)
5. To study hASC potential in angiogenesis induction *in vitro* and to develop hASC angiogenesis *in vitro* assays (III)
6. To create a routine *in vitro* angiogenesis test method for drug and chemical screening by optimizing and validating a scientific research assay (IV)

Materials and methods

Ethical approvals (I-IV)

The use of human tissue in this study was conducted in accordance with the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland (ethical approval R03058 for adipose tissue and R08028 for umbilical cords). The animal experiments were performed according to the Finnish animal protection laws and approved by the Department for Social Welfare and Health Services of State Provincial Office of Western Finland.

Cells and tissue samples (I-IV)

The subcutaneous adipose tissue samples were obtained from surgical operations from female ($n=33$) and male donors ($n=4$) and the human umbilical cords from scheduled caesarean sections ($n=19$) with informed consents at the Tampere University Hospital, Tampere, Finland. The BJ human foreskin fibroblast cell line was purchased from American Type Culture Collection (ATCC).

Reagents, equipment and computer programs (I-IV)

Oligonucleotides for platelet/endothelial cell adhesion molecule-1 (PECAM), Ang-1, Caldesmon, and ribosomal protein large P0 (RPLP0) were purchased from Oligomer Oy, Helsinki, Finland. Lentiviral construct pLKO-MISSION-Bright-GFP was purchased from Biomedicum Genomics, Biomedicum Helsinki, Helsinki, Finland. anti-CD3 (Clone SP7) rabbit monoclonal antibody, DAKO EnVisionTM+ System, HRP kit, DAKO REALTM Peroxidase-Blocking Solution and DAKOCytomation Liquid DAB Substrate Chromogen System were from DakoCytomation Glostrup, Denmark. Milk powder was obtained from Valio Oy, Helsinki, Finland.

Mouse on Rat HRP Polymer Kit and Rodent Block R were from Biocare Medical, Concord, CA, USA. Monoclonal mouse anti rat CD68 and mouse anti human Cd79a were from AbD Serotec, MorphoSys AG, Martinsried, Germany. TRIzol[®] and Collagenase I were purchased from

Invitrogen, Carlsbad, CA, USA. Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 (DMEM/F12), Minimal Essential Medium with Earle's salts w/o L-Glutamine (MEM), antibiotic-antimycotic mixture (AB/AM), non-essential amino acids (NEAA), foetal bovine serum (FBS), L-glutamine and TrypLE Express were from Gibco, purchased from Invitrogen. Histostain Plus Broad Spectrum Detection Kit, DAB Substrate Kit and Digest-All™ 2 kit were from Zymed Laboratories Inc, purchased from Invitrogen. EGM-2 bullet kit Medium, Endothelial Basal Medium (EBM), EGM-2 SingleQuots-supplements, MycoAlert® Mycoplasma Detection Kit, Gentamicin Sulfate 50 mg/ml and Amphotericin B 250 µg/ml were obtained from Lonza Group Ltd., Basel, Switzerland. Non-animal derived partially cross-linked hyaluronic acid Restylane was from Q-Med, Uppsala, Sweden. Erlotinib was a kind gift from Roche Diagnostics GmbH, Mannheim, Germany.

CD144-PE, CD202b, VEGFR2, Recombinant human bFGF (for study IV), recombinant human VEGF as well as Quantikine Human VEGF Immunoassay, Quantikine Human FGF basic Immunoassay and Quantikine Human IGF-I Immunoassay were obtained from R&D Systems, Abingdon, UK. Bovine Serum Albumin (BSA) Fraction V (for study IV) was from Roche, Indianapolis, IN, USA. Human serum was from Cambrex, East Rutherford, NJ, USA. Human basic Fibroblast Growth Factor (study I and III), biotin, pantothenate, insulin, dexamethasone, isobutyl-methylxanthine (IBMX), anti-von Willebrand Factor (vWf) primary antibody produced in rabbit, monoclonal anti alpha smooth muscle actin (αSMA) clone 1A4, anti-smooth muscle myosin heavy chain (SMMHC), anti-calponin, anti-platelet derived growth factor receptor-β (PDGFRβ), BSA (for study II), polyclonal anti-rabbit IgG TRITC, polyclonal anti-mouse IgG FITC, anti-neurofilament 68 (NF-68) produced in mouse, casein, Neutral Red Solution, anti-vascular endothelial growth factor, thalidomide, levamisole (tetramisole hydrochloride), 2-methoxyestradiol, acetyl salicylic acid, hexadimethrine bromide and paraformaldehyde were purchased from Sigma Aldrich, Manassas, VA, USA. Polyclonal antibody to rabbit IgG FITC was from Acris Antibodies GmbH, Herford, Germany. BCA protein Assay Kit was from Pierce Biotechnology, Rockford, IL, USA. RayBio® Human Cytokine Antibody Array C Series 1000 was purchased from RayBioTech, Inc., Norcross, GA, USA. ABI Prism 7000 sequence detector, SYBR Green PCR Master Mix kit and High-Capacity cDNA Reverse Transcriptase Kit were from Applied Biosystems, Foster City, CA, USA. Biotinylated Anti-Rabbit IgG (H+L) made in goat, and Vectastain Elite ABC Kit were purchased from Vector laboratories Inc, Burlingame, CA, USA. Triton X-100 was from JT Baker, Phillipsburg, NJ, USA. Xylene was from VWR International, LLC, PA, USA and hydrogen peroxide and methanol were from Mallinckrodt Baker Inc., Deventer, The Netherlands. Papanicolauslösung 1a Harris' Hämatoxylinlösung, Entellan and isopropanol were from Merck KGaA, Darmstadt, Germany. Pertex was from HistoLab Products Ab, Gothenburg, Sweden.

The antibodies for flow cytometric analysis; CD13-PE was from BD Biosciences, Erembodegem, Belgium; CD31-FITC, CD34-APC and CD44-FITC were from Immunotools GmbH Friesoythe, Germany; CD117-APC and CD 133-PE from Miltenyi Biotec, Bergisch Gladbach, Germany; and CD63-PE and anti-vWF (for study III) from Abcam, Cambridge, UK. IgG-PE was obtained from CalTag Laboratories, CA, USA.

The 48-well plate inserts were obtained from Scaffdex Oy, Tampere, Finland. 50 ml tubes, 75 cm² flasks (Nunc EasyFlask™) and 48-well plates (Nuncclon™ Multidish) were from Nunc, Roskilde Denmark. IbiTreat 8-well cell culture slides were from IbiDi GmbH, Martinsried, Germany. 100 µm, 40 µm and 0.22 µm filters were obtained from Sarstedt, Nümbrecht, Germany, 96-well optical reaction plates from Applied Biosystems and 1 ml syringes and 27 gauge needles from Terumo Europe, Leuven, Belgium. Bürker Chamber was from Menzel-Gläser, Braunschweig, Germany. Domitor (medetomidin hydrochloride) was purchased from Orion Pharma, Espoo, Finland and Ketalar (ketamin hydrochloride) from Pfizer, Espoo, Finland.

Leica DM 2000 microscope and Leica DM2000 microtome were from Leica Instruments GmbH, Germany and microtome Microm HM 430 from Microm GmbH, Waldorf, Germany. Sony DXC-S500 camera control unit and Sony Twain Driver Software 1.0 were from Sony Europe Limited, Weybridge, Surrey, UK. The chemiluminescence reader FluorChem™ 8800 imaging system and FluorChem™ software v. 3.1. were from Alpha Innotech, San Leandro, CA, USA. Fluorescence activated cell sorter FACSaria was from BD Biosciences, Erembodegem, Belgium. Varioskan Flash Spectral Scanning Multimode Reader was from Thermo Fisher Scientific Inc. Waltham, MA, USA. Nikon Microphot FXA microscope, Nikon Eclipse TS-100 microscope, Nikon DS Camera Control Unit DS L-1 and Nikon Eclipse Ti-S microscope were from Nikon, Tokyo, Japan. Confocal laser scanning microscope Zeiss LSM 700 (Zeiss LSM 700 on the Axio Observer) and Zen2009 confocal imaging software were from Carl Zeiss Microimaging GmbH, Jena, Germany. Canon Photo Stitch 3.1 was from Canon, Tokyo, Japan, Adobe Photoshop softwares 7.0 and CS3 were purchased from Adobe Systems, San Jose, CA, USA, Corel Draw software 10.0 from Corel Corporation, Ottawa, ON, Canada and GraphPadPrism 5.0 from GraphPad Software, Inc., San Diego, CA, USA.

Adipose tissue extract preparation

Human adipose tissue extract preparation for cell culture and animal studies (I,II)

Human adipose tissue specimens were minced into small (approximately 1mm²) pieces. Tissue pieces were transferred into 50 ml tubes. Equal amount (1:1) of DMEM/F12 medium or alternatively, phosphate buffered saline (PBS) was added into each tube and the tubes were left for growth factor extraction at 37°C in a 5%CO₂ incubator. A few of the samples were incubated in room temperature. The tubes were gently shaken a few times during incubation. The extract was then collected at several time points (15 min, 30 min, 1hr, 2hr, 3hr, 4hr, 5hr, 6hr or 24 hr). After incubation time, the tube contents were sentrifuged at 1000 rpm for 3-5 min, and the floating adipose tissue layer was removed with Pasteur pipette. The liquid extract was then removed from the tube, filtered through a 40 µm filter into another tube and centrifuged at 12 000 rpm for 5 min. The obtained supernatant was sterile filtered through 0.22 µm filter and stored in aliquots at -20°C.

Rat adipose tissue extract (rATE) preparation for animal studies (II)

Fat tissue from sacrificed rats was minced into small (approximately 1mm²) pieces and transferred to a 50 ml tube. Equal amount (1:1) DMEM/F12 medium or phosphate buffered saline (PBS) was added into tube and left for growth factor extraction at 37°C in a 5%CO₂ incubator for 24 hours. The extract was then removed from the tube, filtered through a 40 µm filter into another tube and centrifuged at 12 000 rpm for 5 min. The obtained supernatant was sterile filtered through 0.22 µm filter and stored in aliquots at -20°C.

Adipose tissue extract characterization

Protein measurement (I,II)

ATE samples, from different patients or from animals and/or from different time points ($n=80$), were analyzed for the total protein concentration with BCA protein Assay Kit according to the manufacturer's instructions.

Growth factor measurement (I)

The concentrations of VEGF, bFGF and IGF-1 were measured from human adipose tissue extract samples ($n=26-58$) with Quantikine Human VEGF Immunoassay, Quantikine Human FGF basic Immunoassay or Quantikine Human IGF-I Immunoassay according to the manufacturer's instructions.

Cytokine array (I)

Altogether 120 cytokines were tested from six different extract samples at two different time points (1 hr and 24 hrs) with RayBio® Human Cytokine Antibody Array C Series 1000 according to the manufacturer's instructions. The chemiluminescence was detected with FluorChem™ 8800 imaging system and the chemiluminescent signals were measured with FluorChem™ software 3.1. and analyzed qualitatively and semiquantitatively.

Cell isolation and culture

The cells were maintained at 37°C under a 5% CO₂ air atmosphere at a constant humidity and medium was changed every two to three days. A complete list of the cell culture and differentiation media used throughout the study is shown in Table II.

Table II. A complete list of the cell culture and differentiation media used for cell culture studies.

Culture condition/medium	Basal medium	Serum	Supplements	Study
Fibroblast proliferation medium	MEM	10% FBS	NEAA, 1% L-glutamine, 1% GA	I, IV
HUVEC proliferation medium (EGM-2 BulletKit medium)	EBM-2	2% FBS	Single Quots –supplements (0,1% GA-1000, IGF-I, EGF, VEGF, FGF-2, ascorbic acid, hydrocortisone, heparin)	I,III,IV
human ASC (hASC) proliferation medium (Adipogenesis negative control medium)	DMEM/F12	15% HS	1mM L-glut, 1% AB/AM, 15% HS	I,II,III
Angiogenesis negative control medium (basic test medium)	EBM-2	2% FBS	0,1% GA-1000(#), 1% L-glut	I,III, IV
Standard adipogenic medium	DMEM/F12	10% FBS	1% AB/AM, 1% L-glut, 33 µM biotin (Sigma Aldrich, Manassas, VA, USA), 17 µM pantothenate, 100 nM insulin, 1 µM dexamethasone, 0.25 mM IBMX(*)	I
ATE adipogenesis induction medium I	DMEM/F12	15% HS	1mM L-glut, 1% P/S, 350 µg/ml of ATE	I

ATE adipogenesis induction medium II	DMEM/F12	15% HS	1mM L-glut, 1% P/S, 700 µg/ml of ATE	I
ATE adipogenesis induction medium III	DMEM/F12	15% HS	1mM L-glut, 1% P/S, 950 µg/ml of ATE	I
ATE adipogenesis induction medium IV	DMEM/F12	15% HS	1mM L-glut, 1% P/S, 1200 µg/ml of ATE	I
ATE angiogenesis induction medium II	EBM-2	2% FBS	0,1% GA-1000 [#] , 1% L-glut, 900 µg/ml of ATE	I
ATE angiogenesis induction medium III	EBM-2	2% FBS	0,1% GA-1000 [#] , 1% L-glut, 1300 µg/ml of ATE	I
Angiogenesis induction medium I for hASC and HUVEC angiogenesis assay(¤) (EGM-2 BulletKit medium)	EBM-2	2% FBS	Single Quots –supplements (0,1% GA-1000, IGF-I, EGF, VEGF, FGF-2, ascorbic acid, hydrocortisone, heparin)	I,III,IV
Angiogenesis induction medium II for hASC and HUVEC angiogenesis assay (EGM-2 BulletKit medium with human serum)	EBM-2	2% HS	Single Quots –supplements (0,1% GA-1000, IGF-I, EGF, VEGF, FGF-2, ascorbic acid, hydrocortisone, heparin)	I,III,IV
Angiogenesis induction medium III for hASC and HUVEC angiogenesis assay (EGM-2 BulletKit medium w/o serum)	EBM-2	serum free	Single Quots –supplements (0,1% GA-1000, IGF-I, EGF, VEGF, FGF-2, ascorbic acid, hydrocortisone, heparin)	I,III,IV
Angiogenesis induction medium I for BJ+HUVEC angiogenesis assay(\$)	EBM-2	2% FBS	2% FBS, 0,1% GA-1000, 1% L-glut, 1.0 ng/ml VEGF, 0.1 ng/ml bFGF	I, IV
Angiogenesis induction medium II for BJ+HUVEC angiogenesis assay(\$)	EBM-2	2% FBS	2% FBS, 0,1% GA-1000, 1% L-glut, 2.5 ng/ml VEGF, 0.25 ng/ml bFGF	I, IV
Angiogenesis induction medium III for BJ+HUVEC angiogenesis assay(\$)	EBM-2	2% FBS	2% FBS, 0,1% GA-1000, 1% L-glut, 5 ng/ml VEGF, 0.5 ng/ml bFGF	I, IV
Angiogenesis induction medium IV for BJ+HUVEC angiogenesis assay(\$)	EBM-2	2% FBS	2% FBS, 0,1% GA-1000, 1% L-glut, 7.5 ng/ml VEGF, 0.75 ng/ml bFGF	IV
Angiogenesis induction medium V for BJ+HUVEC angiogenesis assay(\$)	EBM-2	2% FBS	2% FBS, 0,1% GA-1000, 1% L-glut, 10 ng/ml VEGF, 1 ng/ml bFGF	IV
Angiogenesis induction medium VI for BJ+HUVEC angiogenesis assay(\$) = Positive control medium, stimulation medium	EBM-2	2% FBS	2% FBS, 0,1% GA-1000, 1% L-glut, 25 ng/ml VEGF, 2.5 ng/ml bFGF	I, IV
Angiogenesis induction medium VII for BJ+HUVEC angiogenesis assay (\$)	EBM-2	2% FBS	2% FBS, 0,1% GA-1000, 1% L-glut, 50 ng/ml VEGF, 5 ng/ml bFGF	IV
Angiogenesis induction medium VIII for BJ+HUVEC angiogenesis assay(\$)	EBM-2	2% FBS	2% FBS, 0,1% GA-1000, 1% L-glut, 75 ng/ml VEGF, 7.5 ng/ml bFGF	IV

(*)IBMX removed from culture after 24 hours, (#)From EGM-2 BulletKit Single Quots – supplements, (\$)BJ fibroblast and HUVEC angiogenesis assay

BJ fibroblast culture (I,IV)

Human BJ neonatal foreskin fibroblasts were cultured in fibroblast proliferation medium as shown in detail in Table II. When cells were grown to confluency, they were subcultured in a ratio of 1:4 to 1:9. The cells were cryopreserved in liquid nitrogen 500 000 cells per vial.

Isolation and culture of human umbilical vein endothelial cells (I,III, IV)

The isolation of umbilical vein endothelial cells (HUVEC) from human umbilical cord veins was performed as described by Jaffe (Jaffe et al. 1973) but the process was further optimized. The umbilical cord was separated from the placenta and the umbilical vein was cannulated with a 20G needle. The needle was secured by clamping the cord over the needle with a surgical clamp. The vein was perfused with Umbilical cord Buffer Solution (UBS; 0.1M phosphate buffer solution containing 0.14M NaCl, 0.004M KCl and 0.011 M glucose) to wash out blood after which the other end of the umbilical vein was clamped with a surgical clamp. The vein was infused with 0.05% collagenase I. The umbilical cord was incubated in a water bath at 37°C for up to 20 min. After incubation, the collagenase solution containing HUVEC was flushed from the cord by infusing the vein with UBS. The suspension was flushed out into a 50 ml polypropylene tube. The cells were centrifuged at 250 x g for 10 min, resuspended in HUVEC proliferation medium and seeded into 75 cm² filtered cell culture flasks. HUVEC were subcultured at primary culture (p0) in the ratio of 1:2 to 1:4 and at passages 1 (p1) upwards in a ratio of 1:3 to 1:5. Cells were cryopreserved in liquid nitrogen mainly at passage 2 (p2), 500 000 cells per vial.

Isolation and culture of human adipose stromal cells (I,II,III)

Human adipose tissue specimens were cut into small pieces and enzymatically digested with 0,05% collagenase I in plain DMEM/F12 for 60 min at 37°C in a gyratory water bath. The digested tissue was centrifuged at 600 x g for 10 min in room temperature. The digested tissue was filtered through a 100 µm filter, centrifuged and filtered through a 40 µm filter. Cells were seeded into 75 cm² flasks in hASC proliferation medium. Cells were subcultured 1:2-1:4 when 70-80% confluent and used for cell differentiation studies or frozen in liquid nitrogen for storage, 10⁶ cells per vial.

Detection of mycoplasma (IV)

BJ fibroblasts and HUVEC were quality-controlled prior to liquid nitrogen storage. The cells were tested for mycoplasma contamination with MycoAlert® Mycoplasma Detection Kit according to manufacturer's instructions.

Determining the cell viability (IV)

The viability of BJ fibroblasts and HUVEC was tested prior to liquid nitrogen storage. Cells were stained with TrypanBlue and the viability was calculated manually with a Burker Chamber under Nikon Ts-100 microscope.

*Adipogenic induction *in vitro* (I,II)*

To initiate adipogenic differentiation, hASC were plated at early passages (p1-p5) at a density of 10 000 cells/cm² into hASC proliferation medium. The next day, six different induction media were applied to hASC. Cells were cultured in the hASC proliferation medium, in the standard adipogenic medium, and in ATE adipogenesis induction media I-IV containing either 350 µg/ml, 700 µg/ml, 950 µg/ml or 1200 µg/ml of ATE. The cell culture media and ATE adipogenesis induction media formulations are shown in detail in Table II. Cells were cultured for 1, 2 or for 4 weeks and examined for lipid accumulation by Oil-Red-O (ORO) staining or used for detecting adipogenic genes with quantitative real-time RT-PCR.

*Angiogenic induction *in vitro**

BJ fibroblast and HUVEC angiogenesis assay (I,IV)

The assay was modified from Bishop et al. (1999) and Friis et al. (2003) and optimized. BJ fibroblasts were seeded in fibroblast proliferation medium at a density of 20 000 cells/cm² into 48-well plates and grown for 3 days. Next, HUVECs were seeded on top of fibroblast cultures at a density of 4000 cells/cm² in HUVEC proliferation medium.

ATE angiogenesis test (I)

The day after plating the HUVEC, the different induction media were applied. Cells were cultured in the angiogenesis negative control medium, in angiogenesis induction media I-VIII and in ATE angiogenesis induction media I-III containing approximately 300 µg/ml, 600 µg/ml or 950 µg/ml of ATE. The cell culture media and ATE angiogenesis induction media formulations used are shown in detail in Table II. The treatments were

applied twice to cells during culture and cells were cultured for six to seven days prior to immunocytochemical analysis.

Optimization of the human umbilical vein endothelial cell passage (IV)

The isolated HUVEC were tested for their tubule formation capacity in the angiogenesis assay from passages 2-10. The BJ fibroblast angiogenesis assay was performed as described above. The day after seeding HUVEC (p2-p10) Angiogenesis induction medium VI for BJ+HUVEC angiogenesis assay i.e. positive control medium, was applied to cells. The cells were cultured for six days prior to DAB-staining.

Linearity and cell batch variation test (IV)

The sensitivity of the BJ fibroblast and HUVEC angiogenesis assay was evaluated by studying linearity and determining the upper and lower limits of detection. Eight different angiogenesis induction media I-VIII for BJ+HUVEC angiogenesis assay (see Table II) were applied to cells. Each induction media was tested in six parallels and the media were applied twice to cells that were cultured for 6 days prior to DAB-staining.

The batch to batch variation between different HUVEC batches was performed to confirm that each of the Master cell bank batches set up in our laboratory gives comparable results. This was done by using different HUVEC cell batches in the linearity test.

Reference chemical test (IV)

The six reference chemicals and their concentrations used for validation of an *in vitro* angiogenesis assay are shown in Table III. The initial concentration range for each chemical was based on our previous studies (data not shown). The selection criteria for the concentrations were: to be in the effective angiogenic range, soluble in the test system, and not to exceed the maximum concentration (0.5%) of dimethyl sulphoxide (DMSO) in the test system. All reference chemicals were prepared in Angiogenesis induction medium IV for BJ fibroblasts and HUVEC angiogenesis assay (positive control, stimulation medium, see Table II).

Table III. The reference chemicals used for intra-laboratory validation of the *in vitro* angiogenesis assay.

Reference chemical/ (Tradename)	Mechanism of action	Use	Concentration range used in the study	Solute
Levamisole (Ergamisol)	phosphatase inhibitor	drug against parasitic worm infections	0.01, 0.1, 1, 10, 50, 100, 250, 500, 750, 1000 and 2000 μ M	DMSO
Acetyl salicylic acid (Aspirin)	non-selective cyclo-oxygenase inhibitor	anti-inflammatory drug (painkiller)	10, 100, 500, 1000, 1500 and 2000 μ M	medium
Thalidomide (Thalomid)	TNF- α inhibitor	anti-cancer drug	10, 100, 200, 300, 400 and 500 μ M	DMSO
Erlotinib (Tarceva)	EGFR tyrosine kinase inhibitor	anti-cancer drug	0.0005, 0.001, 0.01, 0.1, 1, 10, 25 and 50 μ M	medium
Anti-VEGF (Avastin)	VEGF inhibitor	anti-cancer drug	0.5, 1, 2.5, 5, 7.5, 10, 25 and 50 μ g/ml	medium
2-methoxyestradiol (Panzem)	microtubule inhibitor, HIF-1 α pathway inhibitor	anti-cancer drug	0.01, 0.1, 0.2, 0.4, 0.6, 0.8, 1 and 2 μ M	DMSO

hASC monoculture angiogenesis assay (III)

hASC (up to passage 7) were seeded in HUVEC proliferation medium into 48-well plates (Nunc™ Multidishes) at a density of 20 000 cells/cm². Cells were cultured for either 3 or 6 days in EGM-2 BulletKit medium or in DMEM/F-12 medium supplemented with 15% HS, 1mM L-glut and 1% AB/AM. The cell culture media and ATE adipogenic induction media formulations used are shown in detail in Table II. The treatments were applied once to cells cultured for 3 days and twice to cells cultured for 6 days.

hASC and HUVEC angiogenesis assay (III)

hASC (passages 1 to 7) were seeded in HUVEC proliferation medium into 48-well plates (Nunc™ Multidishes) or for confocal imaging into 8-well slides (Ibidi™) at a density of 20 000 cells/cm². HUVEC, cultured as above (up to passage 4), were immediately seeded on top of hASC at a density of 4000 cells/cm² in HUVEC proliferation medium. The day after plating, the differentiation treatments were applied to hASC-HUVEC culture. Treatments were angiogenesis negative control medium, angiogenesis induction media I-III for hASC+HUVEC angiogenesis assay. The media formulations used are shown in detail in Table II. Cells were cultured for either 3 or 6 days prior to immunocytochemistry or quantitative real-time RT-PCR. The treatments were applied once to cells cultured for 3 days and twice to cells cultured for 6 days.

Lentiviral infection (III)

The lentiviral pLKO-MISSION-Bright-GFP infection was carried out with HUVEC at passages 2-4 with 300 µl of pLKO-MISSION-Bright-GFP in 1 ml HUVEC proliferation medium (1 U/ml). Viral infection was accelerated with 8 µg/ml hexadimethrine bromide. After 24 hours of incubation, medium was replaced with fresh medium. Highly fluorescent clones were selected with cloning rings and further with dilution cloning to obtain pure GFP-HUVEC-cultures. After expanding the infected HUVEC, they were used for hASC and HUVEC angiogenesis assay as described above.

Flow cytometric analysis (III)

The HUVECs at passage 3 were analyzed with a fluorescence activated cell sorter (FACS). The list of the surface markers used is shown in Table IV. 10,000 cells were analyzed per sample and the positive expression was defined as the level of fluorescence greater than 99 % of the corresponding unstained cell sample.

Table IV. List of surface markers used in flow cytometric analysis

Surface marker	Description
CD13	PE-conjugated monoclonal antibody against human CD13/Aminopeptidase N
CD31	FITC-conjugated monoclonal antibody against human CD31/PECAM
CD 34	APC-conjugated monoclonal antibody against human CD34
CD44	FITC-conjugated monoclonal antibody against human CD44/hyaluronate, HCAM
CD117	APC-conjugated monoclonal antibody against human CD117/c-kit, SCFR
CD 133	PE-conjugated monoclonal antibody against human CD 133/AC133
CD144	PE-conjugated monoclonal antibody against human CD144/VE-Cadherin
CD202b	monoclonal antibody against human Tie-2/TEK
VEGFR2	monoclonal antibody against human VEGF receptor 2
CD63	PE-conjugated monoclonal antibody against human CD63/Tetraspanin
vWF/factor VIII	monoclonal antibody against human von Willebrand factor

Cytotoxicity test (IV)

The cytotoxicity test was performed prior to reference chemical study for BJ fibroblast and HUVEC *in vitro* angiogenesis assay to assure that only non-toxic concentrations of chemicals were used in chemical exposure. The cell viability was tested with the Neutral Red Uptake (NRU) assay. 24 hours after the establishment of fibroblast and HUVEC angiogenesis assay, the cells were treated with chemical exposure for 24 hours. After chemical

exposure (see Table III for chemical exposure details), the cells were washed with preheated Phosphate Buffered Saline (PBS). 250 µl Neutral Red (NR) medium (25 mg NR/1ml medium) was added into the wells and incubated for 2 hours at 37°C. After incubation, the cells were washed with PBS. After that, 100 µl NR-desorption medium (50% EtOH, 1% acetic acid in H₂O) was added into the wells and incubated in a shaker for 20 min, protected from light. After shaking, the cells were allowed to settle down for 5 min. The absorbance was measured at 540 nm with Thermo Scientific Varioskan Flash Spectral Scanning Multimode Reader.

Quantitative RT-PCR (I,III)

Primers used for angiogenesis and blood vessel maturation or adipogenesis are shown in Table V. The total RNAs from hASC monoculture angiogenesis assay or from hASC and HUVEC angiogenesis assay were extracted from 3 days or 6 days differentiated confluent cultures using TRIzol® following the manufacturer's protocol. Alternatively, total RNA was extracted from hASC adipogenesis culture at 7 days and at 28 days. cDNA was synthesized using High-Capacity cDNA Reverse Transcriptase Kit according to manufacturer's instructions. Quantitative RT-PCR analysis was performed in a 96-well optical reaction plate using an ABI Prism 7000 sequence detector. The RPLP0 gene was used as reference gene for data normalization. Reactions were performed using SYBR Green PCR Master Mix kit, 50 ng cDNA sample and 10 µM primers. The PCR conditions were: 10 min at 95 °C, followed by 40-45 cycles of 15 seconds at 95 °C and 60 seconds at 60 °C. RNA expression was calculated in comparison to RPLP0 RNA expression for cell culture experiments using the Pfaffl method (Pfaffl 2001):

$$\text{ratio of relative expression} = \frac{(E_{\text{target}})^{\Delta C_P \text{ target (control-sample)}}}{(E_{\text{ref}})^{\Delta C_P \text{ ref (control-sample)}}}$$

Table V The list of primers used for quantitative RT-PCR

Primer	Forward	Reverse	Study
RPLP0	5'-AATCTCCAGGGGCACCATT-3'	5'-CGCTGGCTCCCACTTTGT-3'	I, III
PPAR γ 2	5'-CAGTGTGAATTACAGCAAACC-3'	5'-ACAGTGTATCAGTGAAGGAAT-3'	I
ACBP	5'-GCAACTGTGGGCGACATAAA-3'	5'-GGCCTTGCCCGTGAAGT-3'	I
CD31/ PECAM	5'-TCATTCTGGGATCCATATGCA-3'	5'-TGGGTGTAGAGAAGGATTCCGT-3'	III
Ang-1	5'-AGCTACCACCAACAACAGTG-3'	5'-CAAAGATTGACAAGGTTGTGG-3'	III
Caldesmon	5'-AAGAATCCTTGGGACAGGTGAC-3'	5'-GTGGTGGTTGTCTTGGCCTC-3'	III

Implantation studies (II)

Preparation of implants

The adipose tissue extract, either from 1) human (hATE) or from 2) rat (rATE) was mixed with hyaluronan hydrogel in a ratio that the implant contained 57% ATE and 43% HA hydrogel. As a control implant, HA with incorporated plain PBS was used, with corresponding volumes of PBS (57%) and HA (43%). The final ATE concentration in the hATE implant was 1.48 mg/ml of hydrogel (148 µg in each implant) and in the rATE implant was 0.75 mg/ml of hydrogel (75 µg in each implant).

Protein release from implants

The protein release from the implants was tested by incubating the implants in PBS at 37°C up to three weeks. The 48-well plate inserts were filled with HA-ATE hydrogels and put on top of PBS filled 48-well plate culture wells. Aliquots of PBS were collected and analyzed for total protein concentration with BCA protein Assay.

Adipose stromal cell differentiation in the presence of ATE-hyaluronic acid hydrogel

To study the adipogenic effect of bioactive implants in cell culture, the adipose stromal cells were plated at 48-well plates early passages (p1-p3) at a density of 10 000 cells/cm². Cells were seeded and cultured in the hASC proliferation medium. The 48-well plate inserts were filled with HA-ATE hydrogels and put on top of PBS filled 48-well plate culture wells. The medium was changed twice during the study. Cells were cultured for 6 days and examined for lipid accumulation with ORO staining.

Animal model of acellular soft tissue induction

26 male Sprague-Dawley rats (weight 325±75 g, average age of 8 weeks) were housed per 2, 12 hours light and 12 hours darkness, fed pelleted diet and water *ad libitum*. Rats were anesthetized with a mixture of Domitor (0.5 mg/kg) and Ketalar (75 mg/kg). Three different types of hyaluronic acid implants, each incorporated with different substances, were created as described above. 100 µl of the resulting gel mixture was injected with a 1 ml syringe and 27 gauge needle into dorsal subcutis of rat. Implants were injected in between the upper subcutis and muscle layer into rat dorsal subcutis. *Three* constructs per rat were implanted for three rat per time

point ($n=3$ in each time point). The places of implants were varied in different time points. Implants were left under subcutis for 1, 4, 12, 20 or 40 weeks after which animals were sacrificed. The remaining implant and its surrounding tissue was carefully cut from the implant site and further processed for histological analysis.

Cytological stainings

Oil-red-O staining (I)

Lipid accumulation was assessed at six days after the onset of the differentiation experiment by using ORO staining. hASC were fixed with 4% paraformaldehyde for 20 min and rinsed several times with PBS. 0.5% ORO stock solution was prepared in 100% isopropanol and for staining diluted 3:2 in distilled water, left at RT for 10 min and filtered through standard filter paper. hASC were incubated with 60% isopropanol for 2 to 5 min, incubated with the ORO staining solution for 5 min and rinsed several times with PBS. Phase contrast micrographs of the stained cells were taken with Nikon Eclipse TS-100 microscope equipped with Nikon DS Camera Control Unit DS L-1. The images were processed with Adobe Photoshop 7.0 and Corel Draw 10.0.

Immunofluorescence staining (I,III)

The complete list of antibodies used is shown in Table VI. The tubule formation was visualized with endothelial cell specific antibody anti-von Willebrand Factor (anti-vWf). For study I, cells were washed three times with PBS, fixed with ice-cold 70% ethanol for 20 min, permeabilized with 0.5% Triton X-100 for 15 min and blocked for unspecific staining with 10% BSA for 30 min. After blocking, cells were incubated with the primary antibody 4°C overnight. Cells were washed three times with PBS, incubated 30 min with secondary antibody (polyclonal antibody to rabbit IgGFITC, 1:500). Fluorescence was visualized with Nikon Eclipse Ti-S microscope and the images were processed with Adobe Photoshop 7.0 and Corel Draw 10.0.

For study III, to evaluate human adipose stromal cell differentiation, immunofluorescence staining procedure was performed as in study I, except that parallel double immunofluorescence staining with α -vWf was performed. Primary antibody against either common pericytic marker α -smooth muscle actin (α SMA), vascular smooth muscle cell marker smooth muscle myosin heavy chain (anti-SMMHC), contractile smooth muscle cell marker calponin (anti-calponin), pericytic and smooth muscle cell progenitor marker platelet derived growth factor receptor- β (anti-

PDGFR β) or basement membrane marker collagen IV (anti-COLIV) was combined with anti-vWf. Secondary antibodies used were polyclonal anti-rabbit IgG TRITC (1:100) for anti-vWf and polyclonal anti-mouse IgG FITC (1:100) for anti- α SMA, anti-COLIV, anti-PDGFR- β and anti-SMMHC. Fluorescence was visualized with Nikon Eclipse Ti-S microscope or for confocal laser scanning microscope Zeiss LSM 700 and the images were processed with Zen2009 and with Adobe Photoshop 7.0 or CS3 and Corel Draw 10.0.

Table VI The complete list of antibodies used in studies

Primary antibody	Detection/Marker	Dilution rate	Origin	Secondary antibody	Study
calponin	smooth muscle cells	1:800	mouse	IgG-FITC	III
CD79a	B-cells	1:500	mouse	Mouse on RAT HRP Polymer Kit	II
CD68	macrophages	1:100	mouse	Mouse on RAT HRP Polymer Kit	II
CD3	T-cells	1:100	rabbit	Dako EnVision TM System, HRP Kit	II
COLIV	basement membrane	1:500	mouse	IgG-FITC	III
NF-68	neurofilaments	1:500	mouse	Histostain Plus Broad Spectrum Detection Kit	II
α SMA	pericytes	1:200	mouse	IgG-FITC	III
SMMHC	smooth muscle cells	1:800	mouse	IgG-FITC	III
vWf	endothelial cells	1:500	rabbit	IgG-FITC	I
vWf	endothelial cells	1:100	rabbit	IgG-TRITC	III
vWf	endothelial cells	1:5000	rabbit	Biotinylated IgG	IV
vWf	endothelial cells	1:5000	rabbit	Histostain Plus Broad Spectrum Detection Kit	II

DAB –staining (IV)

The media were removed and the cells were washed three times with PBS, fixed with ice-cold 70% ethanol for 20 min, permeabilized with 0.5% Triton X-100 for 15 min and blocked for unspecific staining with 10% BSA for 30 min. After blocking, the cells were incubated with primary antibody (1:5000) at 4°C overnight or for 1-2 hours in RT. Cells were washed three times with PBS, incubated for 30 min with the secondary antibody (Biotinylated Anti-Rabbit IgG, H+L made in goat) and washed again three times with PBS. The cells were then incubated with enzyme conjugate solution (Vectastain Elite ABC Kit) for 30 min, after which substrate was added (DAB Substrate Kit). The color development was followed under microscope for 5 to 10 min and the reaction was stopped with 0.5 M Tris buffer. After staining, 500 μ l of Tris buffer was pipetted into each cell culture well and the plates were sealed with parafilm for storage at 4°C until microscopic analysis for tubule formation.

Histological stainings

Hematoxylin Eosin staining (II)

The histological specimens were cut into 1 mm² pieces and fixed in 4% paraformaldehyde overnight, dehydrated with a graded ethanol series and embedded in paraffin. Specimens were cut into 5 µM thick slices with microtome and stained with hematoxylin-eosin for histology. Briefly, samples were deparafinized by treating them three times for 5 min each with xylene and after that with graded ethanol series. The samples were stained for 10 min with Mayer Hematoxylin or Papanicolauslösung 1a Harris' Hämatoxylinlösung, rinsed with tap water 10 min and with distilled water 2 min, then stained with 1% Eosin for 15 seconds, rinsed with tap water and further with distilled water. The samples were then dehydrated with 94% and 100% ethanol, treated 3 x 5 min with xylene and mounted with Entellan for storage.

Immunohistochemical staining (II)

The complete list of antibodies used is shown in Table VI. The histological specimens were cut into 1 mm² pieces and fixed in 4% paraformaldehyde overnight, dehydrated with a graded ethanol series and embedded in paraffin. Specimens were cut into 3 or 5 µM thick slices with microtome (Leica DM2000, or Microm HM 430, respectively). Samples were deparafinized with xylene 3 x 5 min each, rehydrated with graded ethanol series and washed with distilled water.

For anti-von Willebrand Factor and for anti-Neurofilament-68 staining (anti-vWF produced in rabbit, 1:5000 and anti-NF-68, 1:500, respectively, both 4°C overnight), tissue samples were treated 15 min with 0,5% hydrogen peroxide in methanol, microwave oven -treated 10 min in preboiled 0.01 M sodiumcitrate buffer pH 6.0, samples cooled 20 min in sodiumcitrate buffer and treated with Histostain Plus Broad Spectrum Detection Kit. The color was developed with DAB Substrate kit for 5 min. The samples were counterstained for 5 sec with Mayer Hematoxylin and mounted with Entellan.

For anti-CD3 staining (CD3 (Clone SP7) rabbit monoclonal antibody, 1:100, 90 min at RT), the samples were microwave oven treated 2 x 7 min in 10mM TRIS-HCl 1mM EDTA pH 9.0., cooled for 20 min in TRIS-EDTA buffer and then treated with DAKO EnVision™+ System, HRP kit. For anti-CD79a staining (mouse anti human Cd79a, 1:500, 4 C overnight) and for anti-CD68 staining (monoclonal mouse anti rat CD68, 1:100, in 0.1%BSA in TBS, 4°C overnight), samples were treated with Mouse on Rat HRP Polymer Kit. The color was developed with DAKOCytomation Liquid DAB Substrate Chromogen System, samples were counterstained with

Papanicolauslösung 1a Harris' Hämatoxylinlösung and mounted with Pertex.

The results were analyzed with Leica DM 2000 microscope from five different fields from three animals per each time point. Images were taken with Nikon Microphot FXA microscope connected to a Sony DXC-S500 camera control unit and Sony Twain Driver Software 1.0 and processed with Adobe Photoshop software CS3, Corel Draw software 10.0 and Canon Photo Stitch 2.0.

Microscopic analysis of tubule formation (III,IV)

After immunocytochemical staining, the tubules were analyzed with Nikon Eclipse TS100 microscope from 48-well plate wells with 40x magnification. The extent of tubules in different cultures was quantified visually by using semi-quantitative grading scale from 0 to 10. The analysis and grading was based on tubule formation, the length and the branches of tubules. The tubule formation in different cultures was compared to positive control (Angiogenesis induction medium VI for BJ+HUVEC angiogenesis assay, see Table II).

Analyses of transplanted implant (II)

Determining the capillary density

The number of formed capillaries was calculated microscopically from three animals per time point (each containing three different types of implants), and from five different random high power field (hpf, 400x) in each implant at the implant-tissue interface with Leica DM 2000 microscope. The capillaries were confirmed to be stained positive for vWf, and the capillaries needed to have lumen that was visible in hpf (400x magnification). Both capillary proliferation and larger arteriole- like and venule-like vessels were counted.

Automated quantitative analysis of the adipose tissue formation

In order to quantitate the adipose tissue formation in rat subcutis, an automated computer assisted analysis tool was earlier developed for fat deposit detection. The applicability of the analysis tool for quantification of fat and to evaluating the differences in the fat accumulation between time has been previously described (Ruusuvaari et al. 2009). Briefly, prior

to analysis, the samples were stained as described above in Hematoxylin-Eosin staining. Hematoxylin-eosin stained slides were photographed with Nikon Microphot FXA microscope connected to a Sony DXC-S500 camera control unit and Sony Twain Driver Software 1.0 and analyzed with the analysis tool without any preprocessing to the images. The analysis tool estimates the area of fat coverage by segmenting the hue component of the image with dual thresholding. Size constraints were applied in order to remove small, fractioned areas and excessively large areas, which typically belong to background. Finally, the user was allowed to modify the segmentation result by removing any possible false detections and by adding missing areas. Adding of new areas was implemented using seeded region growing where the user gives the seed point and the area grows around this point (Ruusuvuori et al. 2009).

Evaluation of the local immunological effects of implantation

The local biological effects were quantitatively scored after immunohistochemical staining as described previously (Kotzar et al. 2002; Lehle et al. 2004) and according to the International Organization of Standardization (ISO) Standard ISO-10993-6:2007 (ISO, 2007) when applicable. The response was evaluated (hpf, 400x) from five (5) different fields and from three different animals per time point. The inflammation score criteria were (as modified from ISO 10993-6:2007): 0= no cell type detected, 1= rare, 1-5/hpf (except for giant cells, 1-2/hpf), 2 = 5-10/hpf (except for giant cells, 3-5/hpf), 3 = heavy infiltrate, 4 = packed. The final average irritating ranking (as described in ISO 10993-6:2007) was obtained from the average of overall scores that were multiplied by two for the final irritation ranking. Finally, the results of ATE-implants were subtracted from the results of the control implant to obtain the final score. Final scoring was: non-irritant (0,0 – 2,9), slight irritant (3,0 – 8,9), moderate irritant (9,0 – 15,0), severe irritant (>15).

Validation criteria for angiogenesis in vitro assay (IV)

Criteria for Master cell banks

Quality controlled liquid nitrogen Master cell banks were created from HUVEC and BJ fibroblasts. Quality control (QC) criteria for setting up the Master cell banks were as follows: i) cell cultures had high proliferation capacity, cells were typical and pure in morphology (when microscopically observed prior to Master cell bank establishment) and iii) contained no mycoplasma. No antibiotics were used in cell culture. ii) Cell viability was

over 90%. The BJ fibroblasts were never passaged over 10 times and the passaging of HUVEC was investigated and optimized.

Criteria for the intra-laboratory validation

The technical criteria and validation criteria for the intra-laboratory validation study are shown in Table VII.

Table VII The criteria for intra-laboratory validation

Technical criteria	Accepted value
Positive control value	score 6-7 from 3 different wells, score 5 from one well ¹
Negative control value	always negative (score 0)
Reference chemicals	only one well out of two parallels could be discarded
Validation criteria	Accepted value
Sensitivity of detection	Growth factor curve of each HUVEC batch tested in the BJ fibroblast HUVEC angiogenesis assay must follow linearity, and criteria for the upper and lower limits of detection
HUVEC batch to batch variation	each cell line must follow the criteria for the sensitivity of detection
Precision	total coefficient of variation (CV) $\leq 15\%$ in the positive control value ²
Reliability	the positive control criteria must be met, reference chemicals must give comparable values in each test time (repeatability), the effect of each reference chemical had to be same regardless of the person (reproducibility)

¹The calculated minimum value for positive control 5.75, ²Total variation in the test had to be $\leq 15\%$ when analyzed statistically with coefficient of variation (CV).

Performance

The overall performance of the assay was tested by repeating the identical protocol in a Reference Chemical Test three times by two technicians. The results were analyzed by two different microscopic analysts.

Statistical analyses (I-IV)

Statistical analyses were performed and graphs were processed with GraphPadPrism 5.0. The results were reported as mean \pm SD and differences were considered significant when $p < 0.05^*$, $p < 0.01^{**}$ and $p < 0.001^{***}$.

Study I. Protein concentrations of different ATE samples were subjected to one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test and growth factor concentration measurements to nonparametric Mann-Whitney test. RT-PCR results were subjected to one-

way ANOVA with Tukey's multiple comparisons test. The variation in ATE-induced adipogenesis was analyzed with unpaired t-test.

Study II. Samples were subjected to one-way ANOVA followed by appropriate post-tests. For the capillary formation and adipose tissue formation Tukey's and Dunnett's post tests and for tissue thicknesses Tukey's post test were used.

Study III. Analysis of tubule formation and RT-PCR results were subjected to One-way ANOVA followed by Dunnett's and Bonferroni's post-tests.

Study IV. One-way analysis of variance with Dunnett's post test was used for the statistical analysis of the reference chemical results. The linearity of tubule formation was tested with linear regression and precision with coefficient of variation (CV). The day to day variation of technicians was tested with one-way analysis of variance, person to person variation between technicians with unpaired t-test and person to person variation between analysts with paired t-test.

Results

Protein, growth factor and cytokine content of adipose tissue extract (I)

The protein concentration, the cytokine profile and the concentrations of VEGF, bFGF and IGF-I of ATE were analyzed. The protein concentration of ATE was mainly dependent on incubation time as well as on the ratio of minced fat and extraction solution. The extraction solution (PBS or basal DMEM/F12) or the incubation temperature (room temperature compared to 37°C) did not have effect on protein concentration. The variation in protein concentration did not seem to markedly have impact on the cytokine composition. It was possible to collect aliquots at different time points from one sample, instead of using only one incubation time per sample. In that case, the protein concentration was generally highest at 1 and 2 hours, after which the protein concentration was seen to decrease with time. If one sample was incubated for a prolonged period of time (e.g. 24 to 48 hours), high concentrations of different cytokine compositions could be obtained. The selected total protein concentrations for further analyses (determined with calculated mean) were 1 hr 2.017 mg/ml, (SD=0.5222), 2 hrs 1.783 mg/ml, (SD=0.1516) and 24 hrs 1.120 mg/ml (SD=0.1917).

The concentrations of IGF-I, bFGF and VEGF were measured with enzyme-linked immunosorbent assay (ELISA) at 1 hour (hr) and 24 hours (hrs). The results are shown in Figure in Original Publication I. The IGF-I concentration (pg/ml of protein) was significantly higher at 1 hr than at 24 hrs (1 hr median=709.6, 24 hr median=126.2, $p<0.0148$). The bFGF concentrations (pg/ml of protein) did not vary significantly between time points (1 hr median=217.0, 24 hr median=522.2, $p<0.1031$). The VEGF concentrations (pg/ml of protein) were significantly elevated at 24 hrs compared to 1hr (1 hr median=6.020, 24 hr median=72.36, $p<0.0001$).

In addition, a selection of 120 growth factors and cytokines, known to have effect on angiogenesis, were semiquantitatively analyzed from six different extract samples at two different time points (1 hr and 24 hrs) with RayBioTech Angiogenesis Cytokine Array 1000. In the analysis of the chemiluminescent signals, the protein array results were compared to the negative control in the assay, and the original values were adjusted to the scale from 0-4 (<1=no detectable expression compared to control, 1<1,5 low, $\geq 1,5$ <2,5 moderate, $\geq 2,5$ high, $\geq 3,5$ very high). At 24 hrs, the overall number of cytokines was higher compared to 1 hr time point. Very high at 1 hour

were adiponectin and angiogenin, and at 24 hours adiponectin and IL-6. All of the highly expressed cytokines at 1hr; angiogenin, adiponectin, Ang-2, insulin-like growth factor binding protein 3 (IGFBP-3), TIMP-2 and Fibroblast-associated/Tumour necrosis factor receptor superfamily member 6 (Fas/TNFRSF6), macrophage migration inhibitory factor (MIF), growth regulated oncogene (GRO), were high also at 24 hr. IL-6, leptin, CC-chemokine ligand 5 (CCL5 e.g. RANTES), Ang-2, bFGF, FGF-9, IGFBP-6, TIMP-2, VEGF-D, MIP-1 α , IL-8, tumor necrosis factor related apoptosis inducing ligand receptor 4 (TRAIL R4), IL-12 p40 and macrophage stimulating protein (MSP- α) expressions were mostly increased over time and were very high or high at 24 hrs. Neutrophil activating protein 2 (NAP-2) and angiogenin were quite highly expressed in all samples, but had donor dependent variations in the expression levels between time points. NAP-2 was occasionally were seen to mildly decrease by 24 hours, however, occasionally, vice versa. Angiogenin was high, but sometimes seen to decrease by 24 hrs. Donor dependent variability in the levels of angiopoietin-2, PDGF-B, TIMP-1, leptin, MCP-1 and IGFBP-6 were also found. The only cytokine that had significant increase by 24 hours in the Cytokine Array was IL-6 ($p<0.001$), as tested with one-way ANOVA after semiquantitative analysis of chemiluminescence signals. Moderate or low levels of numerous other cytokines, especially at 24 hrs, were released, as shown in Table VIII. However, cytokines with expression of <1 are considered not to be present at detectable levels in the array. The factors that were quantitatively detected with ELISA: VEGF, bFGF and IGF-1, were detected at low (VEGF), moderate (bFGF) or no expression (IGF-1) in the cytokine array. This indicates that the lowest concentrations of the factors detected in cytokine array were at ng level per mg of total protein. Moreover, no increase in the level of VEGF was detected in the cytokine array.

Table VIII. Cytokines measured with Raybiotech Cytokine Array 1000 and average factor expression by adipose tissue extract at 1 hr and at 24 hrs.

Cytokine	1h	24h	Cytokine	1h	24h	Cytokine	1h	24h
Adiponectin	4,00*	4,0	I-309	0,25	0,8	MIP-3 β	1,00	1,0
AgRP	2,00	2,0	ICAM-1	1,50	2,0	MSP- α	1,75	1,8
Angiogenin	3,50	2,5	ICAM-3	0,75	1,0	MCP-1	0,50	1,0
Angiopoietin-2	2,75	3,0	IFN- γ	0,75	1,0	MCP-2	0,25	0,3
Amphiregulin	1,00	1,0	IGFBP-1	1,25	1,3	MCP-3	0,25	0,3
Axl	1,00	1,0	IGFBP-2	1,00	1,5	MCP-4	0,25	0,5
BDNF	1,00	1,3	IGFBP-4	0,75	0,5	M-CSF	0,75	1,8
BLC	0,5	0,5	IGF-I	0,25	0,0	MDC	0,50	0,8
BMP-4	1,75	2,0	IGFBP-3	3,0	3,0	MIG	0,25	0,5
BMP-6	1,50	1,8	IGFBP-6	2,0	2,3	MIP-1 α	0,50	0,8
BTC	1,50	1,3	IGF-I SR	1,00	1,0	MIP-3 α	0,0	0,5
CCL-28	0,75	1,0	IL-1 R4/ST2	1,25	1,3	b-NGF	0,75	1,0

CK β 8-1	1,50	1,8	IL-1 RI	1,00	1,0	NAP-2	3,25	2,5
CNTF	1,00	0,8	IL-11	1,00	1,0	NT-3	1,25	1,0
CTACK	1,00	1,0	IL-12 p40	2,25	2,0	NT-4	1,00	1,0
Dtk	1,00	1,0	IL-12 p70	1,75	1,0	Osteoprotegerin	1,25	1,0
EGF	1,00	1,3	IL-17	1,00	1,0	Oncostatin M	1,25	1,0
EGF-R	1,50	2,0	IL-2 R alpha	1,00	1,0	PARC	1,75	1,0
ENA-78	1,00	1,0	IL-6 R	1,75	2,0	PIGF	1,25	1,0
Eotaxin	0,75	0,5	IL-8	1,00	1,3	PDGF-BB	0,5	0,8
Eotaxin-2	1,00	1,3	IL-10	1,00	1,3	RANTES	2,25	2,3
Eotaxin-3	1,00	1,0	IL-13	0,01	0,0	SCF	1,50	1,5
Fas/TNFRSF6	3,0	3,0	IL-15	0,0	0,0	SDF-1	0,0	0,3
bFGF	2,0	2,3	IL-16	0,75	1,0	sgp130	1,50	1,3
FGF-4	2,0	1,8	IL-1 α	0,5	1,0	TARC	0,0	0,3
FGF-6	1,0	0,8	IL-1 β	0,25	0,8	TGF- β 1	0,0	0,0
FGF-7	0,5	0,5	IL-1ra	0,75	1,0	TGF- β 3	0,0	0,0
FGF-9	2,25	2,3	IL-2	0,01	0,3	TNF- α	0,75	1,0
Flt-3 Ligand	0,25	0,3	IL-3	0,5	0,8	TNF- β	1,50	2,3
Fractalkine	0,0	0,8	IL-4	0,5	0,8	sTNF RII	1,25	1,0
GCSF	1,00	1,0	IL-5	0,25	0,8	sTNF-RI	1,50	1,3
GCP-2	0,01	0,5	IL-6	0,75	4,0	TECK	0,75	1,0
GDNF	1,00	1,5	IL-7	0,25	0,0	TIMP-1	1,75	2,0
GM-CSF	0,25	0,8	I-TAC	0,75	1,0	TIMP-2	2,5	3,0
GITR-Ligand	2,00	1,8	Leptin	2,00	2,3	Thrombopoietin	1,50	1,8
GITR	2,25	1,8	LIGHT	1,00	1,0	TRAIL R3	1,50	1,5
GRO	2,75	3,0	Lymphotactin	1,75	2,0	TRAIL R4	1,50	2,0
GRO- α	2,00	2,0	MIF	2,50	2,5	uPAR	1,25	1,0
HCC-4	1,00	1,0	MIP-1 α	2,00	2,0	VEGF	1,50	1,3
HGF	1,50	2,0	MIP-1 β	1,00	1,0	VEGF-D	2,25	2,3

Abbreviations: AgRP, agouti-related protein; BDNF, brain-derived nerve factor; BLC, B-lymphocyte chemoattractant; BTC, betacellulin; CCL-28, C-C motif chemokine 28; CK β 8-1 chemokine β 8-1; CTACK, cutaneous T cell-attracting chemokine; Dtk, growth factor receptor tyrosine kinase; EGF-R, epidermal growth factor receptor; ENA-78, epithelial neutrophil activating peptide-78; Flt-3 ligand, Fms-like tyrosine kinase-3 ligand; GCSF, granulocyte colony-stimulating factor; GCP-2, granulocyte chemotactic protein 2; GDNF, glial-derived neurotrophic factor; GM-CSF, granulocyte macrophage colony stimulating factor; GITR-ligand, glucocorticoid-induced TNFR-related protein ligand; GITR, glucocorticoid-induced TNFR-related protein; GRO, growth related oncogene; HCC-4 hemofiltrate C-C chemokine; I-309, T-lymphocyte secreted protein I-309; ICAM-1, intercellular adhesion molecule 1; ICAM-3, intercellular adhesion molecule 3; IFN- γ , interferon- γ ; I-TAC, Interferon-inducible T-cell alpha chemoattractant; LIGHT; M-CSF, macrophage colony stimulating factor; MDC, C-C motif chemokine 22; MIG, monokine induced by interferon gamma; NT-3, neurotrophin 3; NT-4, neurotrophin 4; PARC, C-C motif chemokine 18; SCF, stem cell factor; SDF-1, stromal cell derived factor-1; sgp130, sialoglycoprotein 130; TARC, thymus and activation -regulated chemokine; TECK, thymus-expressed chemokine; TRAIL R3, tumor necrosis factor related apoptosis-inducing ligand receptor 3; TRAIL R4, tumor necrosis factor related apoptosis-inducing ligand receptor 4; uPAR, urokinase plasminogen activator surface receptor; NT-4, neurotrophin 4.

*bold/gray indicates high and/or changed expression level of factor

Differentiation potential of adipose tissue extract in vitro (I)

The adipogenic potential of ATE was studied in cell culture assay and with qRT-PCR. ATE induced differentiation of hASC towards mature

adipocytes. The adipogenic conversion was homogenously seen in hASC from 3 days onwards *in vitro*. Mild adipogenic effect was seen at 125 µg/ml upwards with ATE. The effect increased with longer culture time and higher concentrations of ATE (up to 2000 µg/ml was tested). The variation in adipogenic ability between donors was quantified with ORO extraction after ORO staining of triglycerides. ATE samples containing 1000 µg/ml of ATE ($n=32$) induced 1.5 to 4.1 fold (median 2.28, SD=0.57, CV=24.7%) triglyceride accumulation compared to a standard adipogenic induction medium (Gregoire 2001; Scott et al. 2011). No significant difference in adipogenesis induction was found between ATE samples at 1 hr ($n=17$) and 24 hr ($n=15$) ($p=0.878$, SD=0.3507 and CV=9.84%).

The adipogenesis markers were also studied with qRT-PCR. The key switch of adipogenesis, PPAR γ , was induced between 1 and 4 weeks almost 7-fold with 1200-1500 µg/ml of ATE ($p<0.001$). The expression of late adipogenesis marker ACBP was low in a week (1.7 -fold), but induced 3-fold at four weeks. See Figure 2 in the Original Publication I.

The angiogenic potential of ATE was studied in an *in vitro* angiogenesis assay. The ATE induced time- and dose-dependent induction of endothelial cell tubule formation in an angiogenesis *in vitro* assay. The tubules were developed from day 4 onwards. 7 days after ATE induction, tubule formation was observed at 450 µg/ml or higher concentrations of ATE. 900 µg/ml and 1300 µg/ml of ATE induced moderate to strong induction of tubule formation. The endothelial cell tubule formation at 1300 µg/ml of ATE resembled the tubule induction of angiogenic positive control (see Table II) with 10 ng/ml of VEGF and 1 ng/ml of FGF-2. See Figure 3 in the Original Publication I. However, donor-dependent and time-dependent differences existed. 24 hr time point was occasionally more favourable to angiogenesis than 1 hr time point. Moreover, the effect was not always dose-dependent.

Soft tissue induction with ATE in vivo (II)

The ability of ATE to induce angiogenesis and adipogenesis in tissue was studied by incorporating ATE into hyaluronan hydrogel and implanting the constructs in rat subcutis for 1 to 40 weeks. The implants were initially implanted for 1, 2, 3, 4, 6, 8, 12, 20 and 40 weeks, from which the time points (1, 4, 12, 20, 40 weeks) that presented the most relevant tissue changes, were chosen for further studies.

Induction of capillary proliferation

Capillary induction was evaluated microscopically from histological samples of implants at 1, 4, 12, 20 and 40 weeks and manually quantified

from 5 different microscopic hpf (400x magnification) at 1 and 4 weeks, i.e. prior to adipose tissue accumulation. Both capillary proliferation and larger arteriole-like and venule-like vessels were counted from hematoxylin-eosin -stained histological slides. The histology of capillary proliferation at the implantation site are seen in Figure 4A and 4B. Capillary proliferation with small newly formed capillaries was extensively present at one and four weeks in the implant- tissue interface in ATE -implants. In rATE, also larger arteriole-like and venule-like vessels were seen next to the implant. Control implant did not have any relevant tissue changes. At 12, 20 and 40 weeks, few larger vessels were seen in the control HA implant. However, in ATE- implants at 12, 20 and 40 weeks, especially in rATE-implant, well vascularized, dense, large fat pads, were seen. Capillaries were evenly distributed throughout the fat tissue.

When the capillary count was statistically evaluated, the control implant blood vessel number did not differ between 1 and 4 weeks. hATE-HA -implant induced significantly higher capillary proliferation at 1 week ($p<0.05$) and at 4 weeks ($p<0.001$) compared to the respective control implants. Also, increase in capillary number in rATE-HA -implant compared to control implant was seen, however, the result was not significant. See also Figure 3 in Original Publication II.

Adipose tissue accumulation

The adipose tissue accumulation was visually evaluated from hematoxylin-eosin -stained samples. At week 1 and week 4, cell penetration into all of the implants was seen. Small adipose tissue deposits, indicating triglyceride accumulation, were seen at week 1. Control implant did not show any relevant tissue changes. At 12 and 20 weeks, well vascularized, dense fat pads were seen in rATE and hATE implants. At 12, 20 and 40 weeks, rATE-HA implants had induced large fat pad formations, seen as several layers of fat in tissue (result shown in Figure 4C). At 12 and 20 weeks, modest angiogenesis and fat tissue formation was seen in the control HA implant (see Figure 4D), whereas control tissue had fairly slight fat accumulation (Figure 4F).

The adipose tissue accumulation was quantified with a specific automated computer assisted analysis tool designed for the purpose (Ruusuvaari et al. 2009). The results are seen in Figure 4G. The amount of adipose tissue (% from randomly imaged microscopic field, 40x magnification) was increased during time, however, at 1 and 4 weeks, there was no significant increase in adipose tissue accumulation. At 12 weeks, however, rATE-HA -implant contained significantly more adipose tissue than the same implant at one week ($p<0.05$). At 20 weeks, all implants had more adipose tissue than respective implants at one week (control implant $p<0.05$, rATE implant $p<0.001$ and hATE implant $p<0.001$). Moreover, at 20 weeks, rATE-HA implant and hATE-HA implant had induced more adipose tissue accumulation than control tissue ($p<0.05$ and $p<0.01$,

respectively). At 40 weeks, all implants, as well as control tissue, contained more adipose tissue than respective implants at one week (all significant compared to their respective samples at one week, $p < 0.001$). However, at 40 weeks, rATE-HA and hATE-HA implants had induced more adipose tissue accumulation than control implant (both significant, $p < 0.001$) or what was seen in control tissue ($p < 0.001$ and $p < 0.01$, respectively).

Other histological features of the implant

Nerve bundles, immunostained with anti-neurofilament-68, were detected at 12, 20 and 40 weeks, but seldom at the earlier time points. Fibroblast proliferation and loose connective tissue formation were observed, accompanied with capillary proliferation. This was seen as expanded loose connective tissue formation contributing to the increased tissue thickness. When microscopically observed, control implants had more connective tissue formation and less adipose tissue formation than ATE-implants.

The change in tissue thickness was evaluated by measuring the thickness of the subcutis manually microscopically from histological samples at 40 weeks. The results are shown in Fig. 4E. When statistically evaluated, all implants had induced significant increase in tissue thickness at 40 weeks compared to control tissue (control implant $p < 0.01$, hATE-implant $p < 0.001$, rATE-implant $p < 0.001$). At 40 weeks, the tissue thickness was significantly higher in rATE-implant and hATE-implant (both $p < 0.001$), compared to control implant.

The degradation of control implant was faster than rATE-HA and hATE-HA -implants. At 20 weeks, all implants were equally and partly degraded but still present in tissue. However, at 40 weeks, the control implants were completely degraded. Two of the rATE-HA -implants (out of three animals) were degraded completely, but some implant was still present in one animal. hATE-HA -implant degradation rate was slower than that of other implants, as all of the hATE-HA -implants were still partly present at 40 weeks.

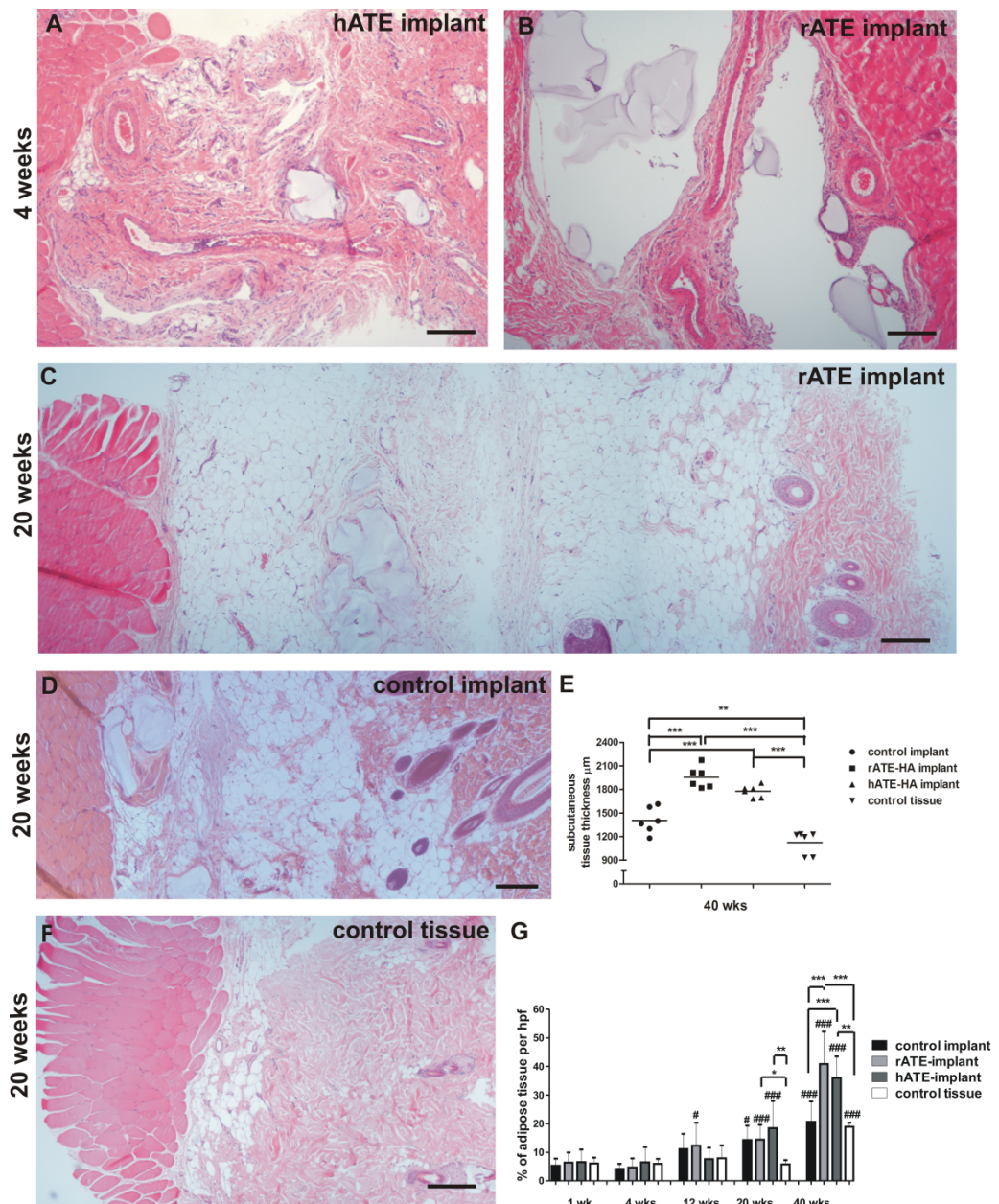


Figure 4. Soft tissue induction with adipose tissue extract *in vivo* (A) Capillary formation in hATE –implant at 4 weeks, (B) Capillary formation in hATE –implant at 4 weeks, Adipose tissue accumulation at 20 weeks in (C) rATE implant, (D) in control implant and (F) in control tissue. (E) The tissue thicknesses in implants at 40 weeks and (G) adipose tissue accumulation during time. The statistical analysis was performed in 4E and 4F as asterisks), as well as in 4G also by comparing each implant to their one week control (significance shown in Fig. 4G as hash signs). Scale bar 200 μ m in each image. Image modified from Original Publication II.

The biocompatibility of the adipose tissue extract (II)

The biocompatibility of the implants was evaluated at 1, 20 and 40 weeks. Implants were seen to be often surrounded by a narrow band of fibroblasts, but after implant was degraded, no signs of fibrous bands were seen. No serious adverse tissue effects occurred. No granuloma, fibrous capsule formation, giant cells or necrosis were seen in any of the samples.

The evaluation of immunological effects of implantation was modified from the International Standardization Organization (ISO) ISO-10993:6-2007 (International Standardization Organization 2007). To be able to perform irritating ranking as described in ISO 10993-6:2007, the active inflammatory changes were multiplied by two, as similarly also described previously (Kotzar et al. 2002; Lehle et al. 2004). The irritation ranking was evaluated solely by immunological effects, not with induction of vascularization or fatty infiltrate, as these were the desired effects of implants. Mild inflammatory cell infiltration was seen in earlier time points. At one week, moderate B-cell (CD79a) and mild macrophage (CD68) infiltration was seen in control implant, as well as mild infiltration of B-cells (CD79a), T-cells (CD3) and macrophages (CD68) in rATE-HA and hATE-HA -implants. The polymorphonuclear cell (evaluated from hematoxylin-eosin staining) infiltration was low throughout the study.

When statistically evaluated, none of the implants were found to be more irritating than others. The results are seen in Figure 5. CD3 (T-cell) positive cell count was significantly higher in rATE-HA implant at 1 week than with any other implant and in two later time points ($p < 0.001$) as well as in hATE-HA implant at 1 week compared to implants at 20 weeks ($p < 0.05$) and 40 weeks ($p < 0.01$). The CD68 (macrophage) positive cell infiltration was significantly higher in control implant at 1 week and at 20 weeks compared to control implant at 40 weeks ($p < 0.01$ and $p < 0.001$, respectively). Moreover, rATE-HA and hATE-HA implants had significantly more macrophage infiltration at 1 week compared to the respective implants at 40 weeks ($p < 0.05$ and $p < 0.01$, respectively). Control implant had significantly more CD79a positive cells at 1 week than at 20 weeks of 40 weeks ($p < 0.05$ and $p < 0.01$, respectively), whereas rATE-HA or hATE-HA implants did not. During time, the number of inflammatory cells was overall decreased and they were completely disappeared at 40 weeks from the animals with control implant, when also the implant itself had degraded completely. rATE-HA and hATE-HA implants were both present at 40 weeks, hATE-HA in each sample and rATE-HA occasionally, and some inflammatory cell infiltration was seen (mild CD79a and mild CD68). No giant cells or necrosis were detected. The total response (shown in Figure 5 as "result") was evaluated by summing up the scores from all of the cells detected. Then the results of rATE and hATE implants were subtracted from the control implant. The implants were found to be non-irritating compared to control implant as evaluated according to ISO (ISO, 2007).

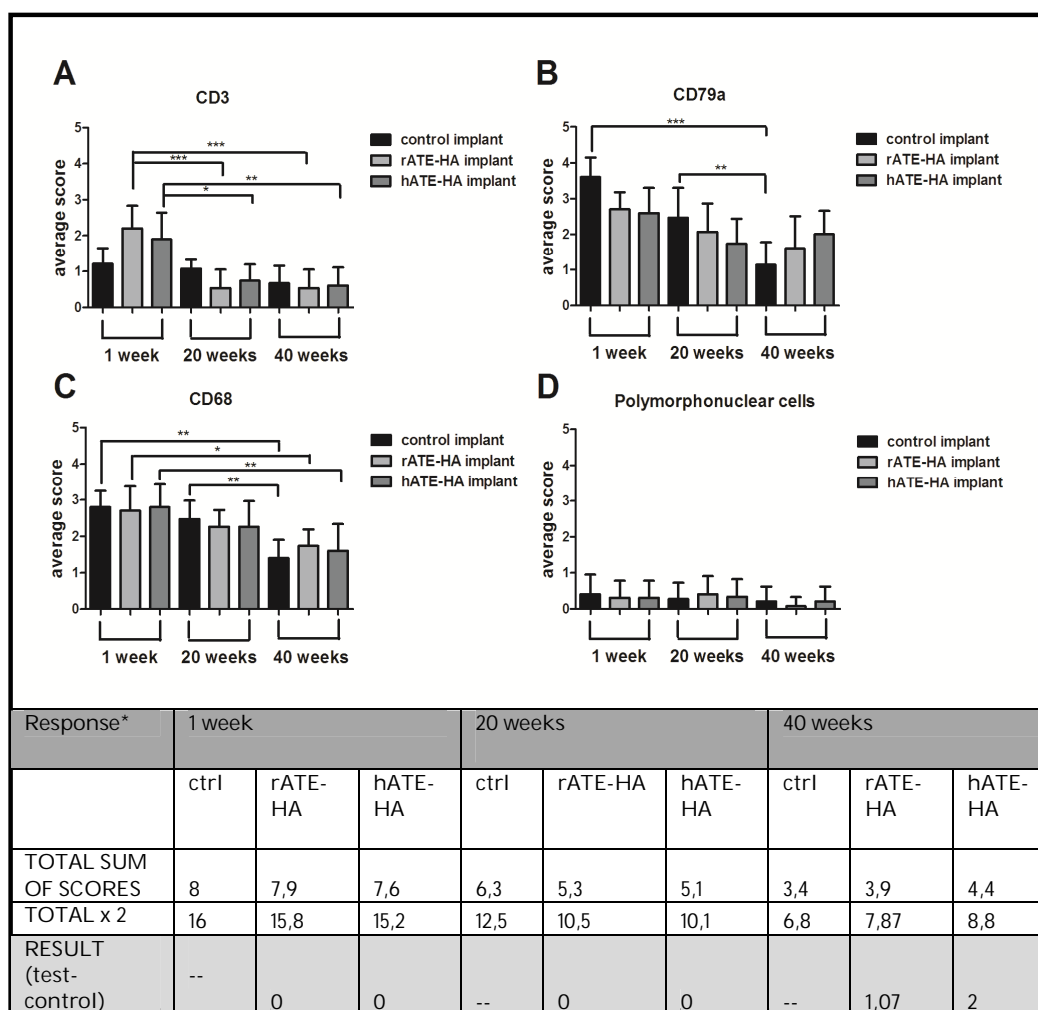


Figure 5. The local effects of implantation. The results are shown as average scores (scale 0= no cell type detected, 1= rare, 1-5/hpf 2 = 5-10/hpf 3 = heavy infiltrate, 4 = packed) from 5 different hpf (400x) and from three different animals per time point. (A) CD3 score, (B) CD79a score, (C) CD68 score (D) polymorphonuclear cell score. The statistical analysis was performed between different implants and by comparing each implant at week 1, 20 and 40. The total response was evaluated by summing up the individual cell scores and finally the results of rATE and hATE implants were subtracted from the result of control implant. The implants were found non-irritating as evaluated according to ISO(2007).

Creation of in vitro assays for angiogenesis (I,III,IV)

Three different angiogenesis *in vitro* assays were studied. The BJ fibroblast and HUVEC angiogenesis assay was optimized and validated to be used as a routine method in drug and chemical screening. Two different hASC assays, hASC monoculture assay and hASC and HUVEC angiogenesis assay

were developed to be used for *in vitro* tissue models and for tissue engineering applications. The morphology of the three *in vitro* assays developed in this study are shown in Figure 6.

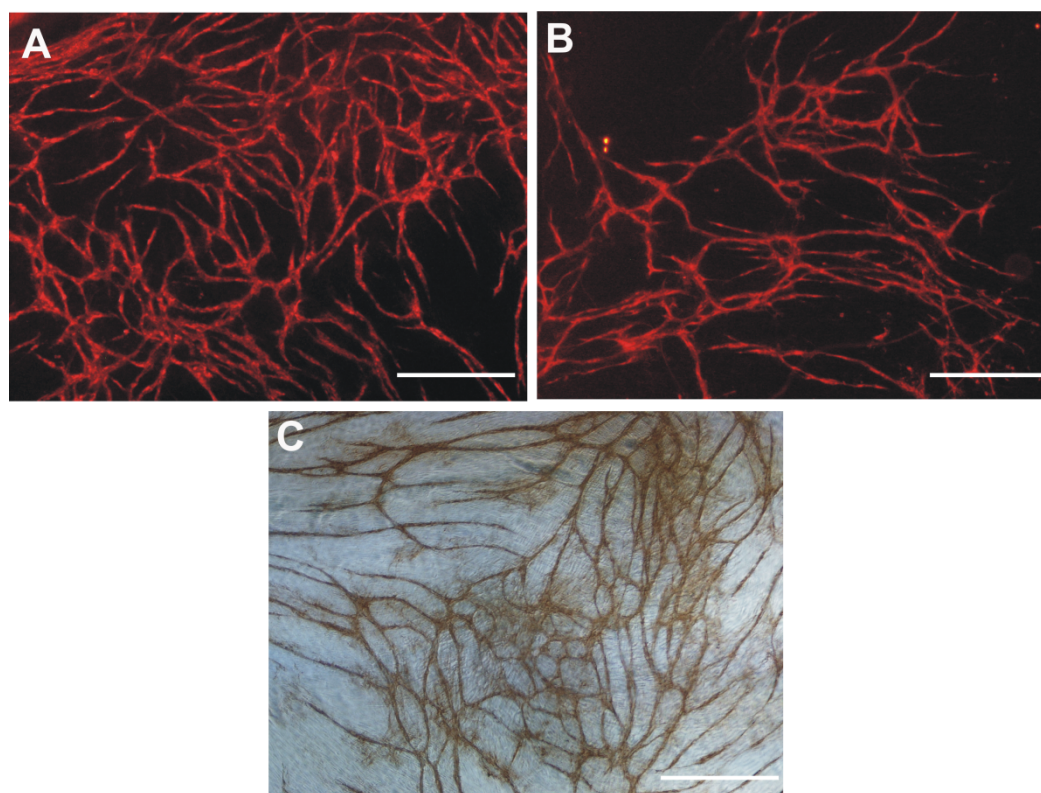


Figure 6. The *in vitro* angiogenesis assays developed in the studies. (A) hASC and HUVEC angiogenesis assay, anti-vWf staining, 1:500 (B) hASC monoculture assay, anti-vWf staining, 1:500 (C) BJ fibroblast and HUVEC angiogenesis assay, anti-vWf staining, 1:5000. Scale bar 500 μ m in each image.

Optimization of the cell banks used in the in vitro assays (III,IV)

The isolated HUVEC were tested for their tubule formation capacity in the BJ fibroblast and HUVEC angiogenesis assay up to passage ten. The tubule formation potency of HUVEC was passage dependent. During cultivation, when the passage number increased, the tubule formation decreased. Up to passage 4 the tubule formation remained constant and at adequate level. The passage number of HUVEC to be routinely used in the BJ fibroblast and HUVEC angiogenesis assay was therefore chosen to be passage 4 (p4) resulting to the passage of 2 (p2) for liquid nitrogen storage. The batch to batch variation of HUVEC was tested with the Linearity and Cell Batch Variation Test. All different HUVEC batches had same lower and upper limits of detection and the technical quality control criteria were accepted with all batches. The variation between the batches was small (CV=1.72%). The flowcytometrically identified surface marker

expression of HUVEC is seen in Table I in the original article III. The surface marker expression profile of HUVEC was mainly corresponding with previous reports (Fukasawa et al. 2006; Ichikawa et al. 2006; Korbling et al. 2006; Schmidt et al. 2006) and expression of e.g. CD13 (99.4%) CD31 (94,5%), CD44 (44,5%), vWf (14,5%) and CD202b (31.9%) were detected.

hASC angiogenesis assays (III)

We induced the tubule network formation in hASC and HUVEC angiogenesis assay or in hASC monoculture angiogenesis assay with naturally occurring angiogenic growth factors; EGF, VEGF, bFGF and IGF-I (Mehta and Besner 2007).

In hASC monoculture, the induction towards angiogenesis was not reproducible between different experiments. hASC monoculture had high passage and donor dependent differentiation capacity towards endothelial-like cells. hASC formed often short cords in culture, but only occasionally tubule network formation. However, vessel supporting pericytic and smooth muscle cell markers were seen quite often, despite the lack of capillary formation at the area.

However, when hASC were combined with HUVEC (hASC and HUVEC angiogenesis assay) and cultured in HUVEC proliferation medium, a remarkable enhancement in cell proliferation, capillary formation and induction of maturation were seen in both cells. hASC and HUVEC formed reproducibly intensive three-dimensional tubular network. The relative mRNA expression of vessel maturation genes *PECAM-1*, *Angiopoietin 1* and *Caldesmon* were studied in both hASC angiogenesis assays. The results of the relative expression levels are shown in Figure 3 in the Original Publication III. *Angiopoietin 1*, a molecule regulating blood vessel stabilization (Nishishita and Lin 2004) and indicating late stages of tubule maturation and stabilization as well as *Caldesmon*, a smooth muscle cell contraction regulator indicating higher differentiation of smooth muscle cells (Frid et al. 1992), were significantly expressed in both hASC and hASC and HUVEC at day 6. However, *Angiopoietin 1* expression was significantly increased by day 6 in both hASC monoculture angiogenesis assays and in hASC and HUVEC angiogenesis assay (both $p < 0.05$) when compared to control. The *PECAM-1* expression was significantly increased between day 3 and 6 in hASC monoculture angiogenesis assay ($p < 0.05$). *PECAM-1* expression was also increased with hASC+HUVEC, but was not statistically significant. *Caldesmon* expression was increased already at day 3 with both hASC monoculture angiogenesis assays and in hASC and HUVEC angiogenesis assay ($p < 0.05$) and even more by day 6 (both assays $p < 0.001$).

In immunofluorescence staining, COLIV, showing the development of basement membrane, was widely expressed in hASC and HUVEC angiogenesis assay. The expression was co-localized with the developing tubules, covering the tubules. In other treatments, basement membrane

formation was incomplete. PDGFR β , an important receptor in pericytic cell recruitment to developing tubules (Nishishita and Lin 2004), was extensively seen already at 3 days as dot-like structures in hASC and HUVEC, localizing at the areas of developing tubules. α -SMA, SMMHC and calponin immunostainings confirmed that the tubules were maturing. The cell coverage with α -SMA, SMMHC and calponin was partial, often located in the tubule branch points. The confocal laser scanning microscopy of basement membrane (COL IV) and tubule network (vWf) staining in Figure 7 shows the three-dimensionality (7A) and multilayered (7B) nature of the tubule network, showing close to 200 μ m thick tubule network with tubules overlaying each other.

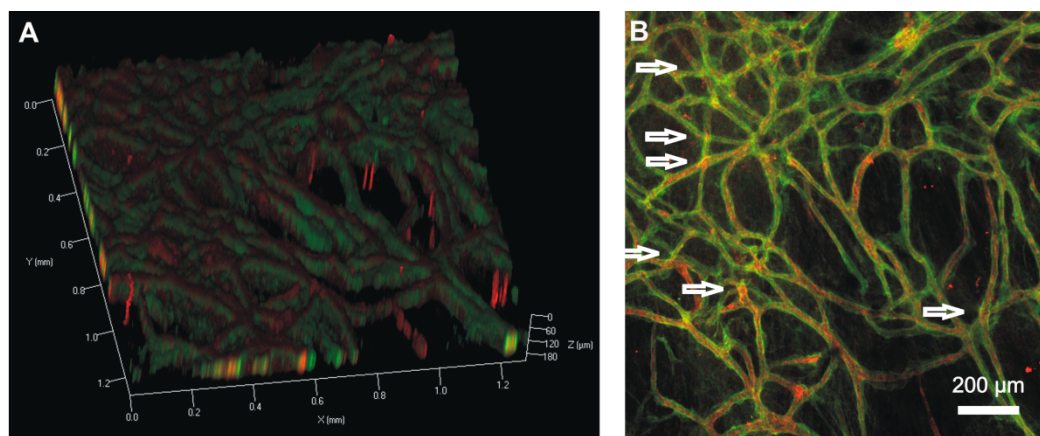


Figure 7. The multilayered tubule network formation in hASC and HUVEC angiogenesis assay. (A) The 3D projection obtained with confocal microscopy showing 196 μ m thick tubule network. (B) The tubule network showing multilayered nature of the network. Tubules crossing each other are pointed out with arrows. Tubule network stained with anti-vWF (1:100) and anti-COL IV (1:100). Image reproduced from Original Publication III.

hASC and GFP-infected HUVEC angiogenesis assay was developed in order to evaluate which cells were responsible for the massive tubule network formation in hASC and HUVEC angiogenesis assay. The anti-von Willebrand factor –stained co-cultures mostly consisted of a mosaic and two different populations of vWf-positive cells, approximately half of the cells in the assay being GFP-positive, and the other half of the anti-von Willebrand factor positive cells being GFP-negative. See Figure 2 in the Original Publication III.

Intra-laboratory pre-validated angiogenesis in vitro assay (IV)

The objective of this part of the study was to intra-laboratory validate the optimized test method for testing the modulators of angiogenesis. The co-culture assay published by Bishop et al. (1999) and Friis et al. (2003) was

further developed, optimized and finally validated in the laboratory in accordance with Organisation for Economic Co-operation and Development (OECD) guidelines (OECD, 2005), The European Agency for the Evaluation of the Medicinal products guideline CPMP/ICH/281/95 (EMA, 1996) and European Centre for the Validation of Alternative Methods (ECVAM) guidance documents (ECVAM, 2002).

Method optimization

In the method optimization phase, the most optimal culture conditions and detection limits for the assay were verified, and the optimal values and criteria for the validation of the assay were determined. The sensitivity of the method was evaluated in Linearity and Cell Batch Variation Test by studying linearity and the upper and lower limits of detection of HUVEC batches. The lowest combination of growth factors that induced tubule formation was a combination of 1 ng/ml VEGF and 0.1 ng/ml FGF-2. This was selected as the lower limit of detection of the assay. The growth factor cocktail that caused maximal tubule formation was a combination of 10 ng/ml VEGF and 1 ng/ml FGF-2. This combination was upper limit of detection and the positive control of the assay. The tubule formation was dependent on the growth factor concentration, and with higher growth factor concentrations than positive control value, the response decreased.

Validation acceptance criteria

The prevalidation phase defines the robustness and intra-laboratory reproducibility of the method and how well it predicts the effects *in vivo* (Curren et al. 1995). In the intra-laboratory validation study, the total variation of the test method was tested by two different technicians and by two different result analysts in three separate tests (referred to as “days” in Table 5 in the Original Publication IV). This part was performed by studying the negative and positive control values, i.e. the predetermined lower and upper limits of the assay. Negative control was always zero (0). The CV% of positive control was found to be between 6.27-7.82%. The CV% between microscopic analysts was very low; CV% was 0.34% when the results of technician 1 were analyzed and 2.37%, when the results of technician 2 were analyzed. The total CV including the total error of the test method was CV= 1.39%. All the results were in the predetermined technical criteria set for positive control. However, the variation in the positive control, that was accepted in the technical criteria, resulted in significant overall person to person variation. The acceptance criteria and the results of the validation study are summarized in Table 5. in the Original Publication IV.

Reference chemical study

A chemical study with the method was performed to test the performance and the reproducibility of the assay. Six reference chemicals, angiogenesis inhibitors, were tested in the assay by two different technicians. The validation criteria were used as acceptance criteria for the test. All the potent inhibitory chemicals selected for the assay inhibited tubule formation, and the results between technicians were in good concordance (see Figure 4 in the original publication IV). The estimated values of concentrations causing mild, moderate or strong inhibition in this assay are summarized in Table IX. The validation of the *in vitro* test system includes the comparison of the efficacy of *in vitro* test with known *in vivo* effects. The results of the chemical test were compared with previous publications on different *in vitro* assays, animal tests and maximal plasma concentrations (C_{\max}) from clinical trials. The results were comparable to other *in vitro* methods, however, the validated method seemed to respond to inhibitors at lower concentrations than previously used *in vitro* assays, especially when acetyl salicylic acid, levamisole and anti-VEGF were evaluated. Compared to selected animal tests, the interpretation of the results was more difficult, but hardly any correlation was detected. Results from clinical trials were in good concordance with acetyl salicylic acid and erlotinib (moderate results in the assay were comparable to C_{\max}), in some extent with levamisole, 2-methoxyestradiol, thalidomide and anti-VEGF (mild inhibition was comparable to C_{\max}). The validated *in vitro* assay seemed more sensitive as inhibitory action was present at lower concentrations, especially with anti-VEGF.

Table IX. The comparison of the inhibitory effects of tubule formation obtained from the reference chemicals in FICAM's assay to the literature data. Table modified from original publication IV.

Reference Chemical	Results from Intra-laboratory Validated Method (inhibition, % from control)			Results from other In vitro Methods	Results from Clinical Studies	Results from Animal Models
	Mild <20%	Moderate 40-60%	Strong 75-85 %	Inhibitory effect and concentration	C_{max}	Effective dose (ED)
Acetyl salicylic acid	1.8–18 µg/ml (10-100 µM)	180 µg/ml (1000 µM)	270 µg/ml (1500 µM)	Moderate 500 µM (Borthwick et al. 2006)	<ul style="list-style-type: none"> 260 µM – 1026 µM (Juarez Olguin et al. 2004) 0-25 µg/ml (Maalouf et al. 2009) 170 ± 96.7 ng/ml (Bae et al. 2008) 	<ul style="list-style-type: none"> 26 µM - 300 µM, CAM model (Sharma et al. 2001)
Erlotinib	0.04–40 ng/ml (0.5 nM- 0.1 µM)	4 µg/ml (10 µM)	22 µg/ml (50 µM)	Mild 1-20 µM (Birle and Hedley 2006) Moderate 10 µM (Jimeno et al. 2007)	<ul style="list-style-type: none"> 0.3 – 1.13 µg/ml (Herbst et al. 2005) 0.251 – 10.7 µg/ml (Ranson et al. 2010a) 2.93 ± 1.3 µg/ml (Ranson et al. 2010b) 0.3 µM (Clarke et al.) 0.56 – 4 µM (Kraut et al. 2011) 	<ul style="list-style-type: none"> 50 mg/kg, Mouse carcinoma model (Jimeno et al. 2007) 50 mg/kg, Mouse tumor model (Cerniglia et al. 2009)
Levamisole	2–240 ng/ml (0.01-1 µM)	25–120 µg/ml (100–500 µM)	240-500 µg/ml (1000 – 2000 µM)	Mild 500 µM, moderate 750-1000 µM, strong 2000 µM (Friis et al. 2005)	<ul style="list-style-type: none"> 0.62 µg/ml – 1.27 µg/ml (Reid et al. 1998) 0.716 ± 0.217 µg/ml (Kouassi et al. 1986) 	<ul style="list-style-type: none"> 1.2 mg/kg - 12 mg/kg, Nude mouse tumor model (Friis et al. 2005) C_{max} 0.37 µg/ml calf parasite infection (Taylor et al. 1988)
2-Methoxy-Estradiol	3–60 ng/ml (0.01 -0.2 uM)	300 ng/ml (1 µM)	600 ng/ml (2µM)	Mild 10 µM, moderate 50 µM (Kang et al. 2006) Mild 0.5 µM, moderate 1 µM (Dobos et al. 2004)	<ul style="list-style-type: none"> 3.3 ng/ml (Tevaarwerk et al. 2009) 30.27± 20.18 ng/ml (Matei et al. 2009) 3.0 -21.4 ng/ml (Dahut et al. 2006) 1.4 – 13.2 ng/ml (James et al. 2007) 2.2 – 9.6 ng/ml (Sweeney et al. 2005) 	<ul style="list-style-type: none"> 100 mg/kg Murine rheumatoid arthritis model (Plum et al. 2009) 7.5 mg/kg, 75 mg/kg Mouse tumor model (Dobos et al. 2004)
Thalidomide	2–25 µg/ml (10-100 µM)	77–100 µg/ml (300-400 µM)	-	-	<ul style="list-style-type: none"> 2 µg/ml (Kakimoto et al. 2002) 1.68 ± 0.41 µg/ml (Murakami et al. 2009) 1.44 ± 0.50 µg/ml (Kamikawa et al. 2006) 0.43 – 1.03 µg/ml (Vieira and Valente Mdo 2009) 	<ul style="list-style-type: none"> 100 mg/kg, Rat Alzheimer model (Ryu and McLarnon 2008) 19 µM - 1000 µM, CAM model (Sharma et al. 2001)
anti-VEGF	0.01-0.1 µg/ml	0.5–1 µg/ml	25-50 µg/ml	Strong 0.1 mg/ml-10 mg/ml (Sims et al. 2008)	<ul style="list-style-type: none"> 363 µg/ml (Herbst et al. 2005) 123.2±16.4 µg/ml (Wu et al. 2010) 11.94 – 194.08 µg/ml (Ning et al. 2010) 20.7 – 24.2 ng/ml (Sharma et al. 2010) 16.6 - 42.5 µg/ml (Krohne et al. 2008) 2.63 - 165 µg/ml (Zhu et al. 2008) 	<ul style="list-style-type: none"> 2-4 mg/kg, Mouse carcinoma model (Sims et al. 2008) C_{max} 104.46 ± 1.44 ng/ml, injection in rabbit (Kim et al. 2010) C_{max} 676 ± 100 µg/ml, cynomolgus monkeys (Xu et al. 2008)

Discussion

There is an urgent clinical need for adipose tissue substitute that would induce rapid vascularization and sustained formation of adipose tissue (Choi et al. 2010). Patients with soft tissue defects may have scars, defects and other abnormalities as well as impaired tissue function that affects the patients in their everyday life, as well as emotionally (Patrick 2001). Treating of the soft tissue defects and chronic wounds are a medical and economic challenge to health care (Ferreira et al. 2006; Levin and Condit 1996). It has been estimated that approximately 5 million U.S. chronic wound patients generate annual over \$20 billion costs for health care and the costs are growing 10% every year (Gordois et al. 2003). In Australia, wounds are estimated to cost \$2.6 billion a year, and in Germany, the costs for care and treatment of pressure ulcers are between €1-2.3 million (Muller-Buhl et al. 2012). Moreover, according to American Society for Plastic Surgeons (ASPS) 13.8 million cosmetic plastic surgery procedures and 5.5 million reconstructive plastic surgery procedures were performed in the U.S. in 2011, with a total rise of 87% in cosmetic procedures during the last decade (ASPS, 2011).

Adipose tissue engineering has traditionally focused on restoring the volume loss with not much emphasis on tissue function (Vermette et al. 2007). Tissue engineered fat *in vitro* and *in vivo* has been studied tremendously during the last decade. However, there are still no universally accepted *in vitro* or *in vivo* models for neoadipogenesis (Bucky and Percec 2008). The main reason for the failure of all the current soft tissue applications is inadequate angiogenesis (Torio-Padron et al. 2007; Vermette et al. 2007). Impaired angiogenesis leads to failure of healing of ulcers, wounds, and infarcted tissue (Cao 2010; Fan et al. 1995). The successful induction of vascularization is therefore a key factor in successful tissue engineering. Solely revascularization of ischemic tissues would benefit millions of people (Carmeliet and Jain 2011).

Clinically relevant *in vitro* angiogenesis models are needed for tissue engineering applications, but also for investigation of the *in vivo* angiogenesis process (Lai et al. 2009; Ucuzian and Greisler 2007) e.g. for the treatment of cancer, macular degeneration or peripheral and coronary vascular diseases (Nillesen et al. 2007; Ucuzian and Greisler 2007). Understanding the role of angiogenesis in adipose tissue is of especial importance, as vasculature regulates both the adipose tissue mass development and adipose tissue reduction and obesity is a cause of a destruction in normal adipose tissue homeostasis (Rupnick et al. 2002).

Since no adequate application for soft tissue restoration nor optimal *in vitro* assays exist, the main aim of the current study was to study induction of angiogenesis and adipogenesis. In the first part of the study, a novel acellular angiogenic and adipogenic agent was produced and its effect was studied *in vitro* and *in vivo*. The angiogenesis induction for the tissue engineered constructs is a prerequisite and a solution is urgently needed. Moreover, the creation of reproducible and relevant *in vitro* angiogenesis assays aids in the angiogenesis research, but also in the development of implantable tissue engineered products. Therefore, the second part of the study focused on developing *in vitro* methods for angiogenesis induction. In the following chapters, the main findings will be discussed.

The cytokine content of adipose tissue extract

As adipose tissue is known to secrete numerous cytokines and to have a role as an active endocrine organ, the freshly isolated adipose tissue is suspected to be the most potential natural source of bioactive factors for inducing angiogenesis and adipogenesis (Nillesen et al. 2007; Saiki et al. 2006). The growth factor and cytokine content of the adipose tissue extract revealed that ATE contains all the relevant angiogenesis stimulating (e.g. VEGF, IGF-I, bFGF, angiogenin, leptin, HGF, IL-6, IL-8) factors (Christiaens and Lijnen 2010; Ziche et al. 2004), main adipokines (adiponectin, leptin) and a wide variety of other regulators of angiogenesis and adipogenesis (e.g. Ang-2, TIMPs). The cytokine profile of adipose tissue extract largely corresponds to previous reports that have studied hASC cytokine release (Verseijden et al. 2009; Rehman et al. 2004; Kilroy et al. 2007; Traktuev et al. 2008; Rubina et al. 2009) or cytokine release from adipose tissue explants (Fain et al. 2004). In addition, IFN- γ , TNF- α , IL-8, bNGF, MCP-1, MCP-2 and leptin, known to be secreted by macrophages or mature adipocytes, were detected at high or moderate levels in the current study. In contrast to Fain et al. (2004) adipose tissue explant study, the cytokine levels of ATE were mainly increased during time, only two angiogenesis inducers, NAP-2 and angiogenin (Gao and Xu 2008), occasionally possibly seen to decrease. NAP-2 contributes to early inflammatory process (Petersen et al. 1994) and vascular regeneration after injury (Gleissner et al. 2008). Angiogenin is reported to be elevated in obesity (Silha et al. 2005). Nevertheless, although the overall number of cytokines was increased over time, only two factors, VEGF and IL-6, were significantly increased during incubation. Increase in VEGF and IL-6 expression indicates possible induced angiogenic potential of the 24 hr extract. The 24 hr rise in VEGF may be due to increase in IL-6, which is known to enhance VEGF production in adipose tissue (Rega et al. 2007). Moreover, hypoxic conditions, which may have occurred during ATE incubation, contribute to increase in VEGF production (Rehman et al.

2004). Roughly, overall, according to the main factors present in ATE, ATE is a proliferation inducing (bFGF, GRO), angiogenic (IL-6, VEGF-D, bFGF, leptin, angiogenin, angiopoietin-2, GRO, MIF, NAP-2) and adipogenic (leptin, bFGF, IGFBP-6, adiponectin) agent, that is also capable of activating further cytokine production in tissue (Fas/TNFRS6, IL-6, MIF). The clinical use of high concentrations of inductive growth factors can lead to unwanted side effects, such as abnormal vascular function or stimulation of tumor growth (Brey et al. 2005; Epstein et al. 2001) and has led to withdrawal of growth factor related drugs from the market. It is noteworthy, that also several endogenous angiogenesis/adipogenesis inhibitors were detected in ATE (such as TIMPs), which suggests that a correct interplay of factors in tissue remodeling can be achieved with ATE, suspectedly preventing any excess stimulatory effect and thus increasing the clinical safety of this material.

It has become evident that obese individuals secrete more inflammatory cytokines than lean individuals (Fain et al. 2004) and that the excess cytokine production has inductive effects on adipose tissue accumulation. As adipose tissue samples are commonly obtained from plastic surgery resections or from liposuctions, it must be kept in mind that some of the patients may have increased BMI and therefore the extract cytokine profile might reflect the donor BMIs. Increase in IL-6, IL-10, MIF, MIP-1 α , TIMP-1, TIMP-2, TECK, ENA-78, PARC, VEGF-A, -C and -D, Ang-2, HGF and angiogenin in obese individuals has been reported (Fain et al. 2004; Silha et al. 2005; Skopkova et al. 2007). Moreover, higher levels of adiponectin, leptin, HGF (Fain et al. 2004) and RANTES (Skopkova et al. 2007) have been reported in lean individuals. On the other hand, adiponectin, TNF α and IL-6 have also reported to remain stable between lean and obese states (Skopkova et al. 2007). In ATE, adiponectin, leptin, and RANTES were expressed at quite high levels. This, along with the possible decrease in NAP-2 and angiogenin, and low IL-6 and VEGF in the 1 hr time point, indicate that the cytokine content of ATE seem not to correlate to, or have a profile of obese adipose tissue or chronic inflammation. It is also noteworthy, that leptin and adiponectin, as circulating hormones, are mainly released by adipose tissue to the body (Fain et al. 2004). Therefore, they are secreted at considerably high levels from adipose tissue compared to most other factors. However, as there is increasing knowledge on the importance of appropriate combination of factors for inductive angiogenesis/adipogenesis, the paracrinically acting factors, although secreted at much lower levels than circulating hormones, are invaluable in contributing to the effects obtained with ATE. Therefore, the changes in the overall composition of ATE are assumably more important than the actual concentrations of factors.

Adipose tissue extract is an angiogenesis and adipogenesis inducing agent *in vitro*

The ability of ATE to induce angiogenesis and adipogenesis were studied in respective *in vitro* assays. ATE was shown to induce accelerated and significantly higher triglyceride accumulation in hASC compared to commonly used standard adipogenic treatment (Gregoire 2001). The strongly adipogenic nature of ATE was also seen as increased expressions of ACBP, and especially, key inducer of adipogenesis, PPAR γ 2, superseding the standard IBMX induced adipogenic treatment both morphologically and genetically. The most striking finding is that contrary to a standard adipogenic treatment, a vast majority of the cells homogeneously differentiated towards mature adipocytes even at low concentrations, and moreover, no contact inhibition for triglyceride accumulation was required. The strongly adipogenic nature of ATE *in vitro* is not surprising, and it directly reflects the source and cytokine profile of ATE. ATE is therefore useful for modeling natural adipogenesis *in vitro*.

The ATE-induced endothelial cell tubule formation *in vitro* was morphologically similar to the VEGF and bFGF induction. Angiogenesis induction was achieved with moderate protein levels, and as this induction was time-dependent, it was, although also dose-dependent, still more composition-dependent. The possible candidates for the increased angiogenic capability of the later time point are VEGF and IL-6, the only two factors that were significantly increased during incubation. The angiogenic nature of ATE in *in vitro* angiogenesis assay is moderate. This seems to be one advantage of ATE. No excess angiogenesis was seen *in vitro*.

Adipose tissue extract induces sustained soft tissue formation *in vivo*

No adequate information on the long-term stability and inductibility of different adipose tissue engineering strategies exists. Previous studies show some adipose tissue formation but no vascularization (Hemrich et al. 2005; Tsuji et al. 2009), limited longevity of the adipose tissue or lack of information on long-lasting (over 3 month) effects (Borzacchiello et al. 2007; Hemrich et al. 2005; von Heimburg et al. 2003). The specific question in the current study was whether ATE could be inductive in soft tissue without cell incorporation and second, whether the effect could last in the implantation site after biomaterial degradation.

The extensive capillary proliferation early after implantation was evident. The induction of vascularization *in vivo* during the first four weeks was well in line with the protein release rate of the implants detected in

our study *in vitro*. As with *in vitro* studies, the angiogenesis/adipogenesis effective concentrations of ATE were fairly low, suggesting that the orderly combination of factors is truly more important than high concentrations of factors. The concentrations of inductive factors should rather mimic naturally occurring concentrations.

The histology of the newly formed adipose tissue resembled that of endogenous adipose tissue when observed at late time points. It is an important finding that ATE is able to induce efficient tissue restoration with normal vascular remodeling. No excess angiogenesis was seen in the formed fat pads *in vivo*. Nevertheless, the adipose tissue volume and tissue thickness were increased several folds in ATE-implants compared to control tissue and control implant, and the formed fat pads were seen to remain in tissue until the follow up time (9 months). The sustained effect in this extent, and for this period of time, has not been demonstrated previously by any of the applications, neither with hASC seeded scaffolds nor with acellular scaffolds.

The timing of adipose tissue appearance in ATE-implants most likely reflects the release properties of the crosslinked hyaluronic acid used in the study. The inductive factors are first released by diffusion from the implant, and later simultaneously with the degradation of the material. The particular hyaluronic acid is clinically used soft tissue filler, designed to be a slowly-degrading material in tissue. The inductive effect could be possibly accelerated with a selection of a different type of biomaterial. Moreover, the concentration dependency of the extract shown in *in vitro* studies indicates that even higher inductive effect could be possibly reached with increased concentrations of inductive extract. It is also noteworthy, that tissue fillers like hyaluronic acid are known to contribute to angiogenesis and adipogenesis (Hanson et al. 2011; Hemmrich et al. 2008; Ventura et al. 2007) in tissue. This was seen in this study with the specific control tissue filler as an increase in the tissue thickness and adipogenesis compared to normal rodent tissue. The tissue increase in control tissue filler, was, however, often contributed by connective tissue formation induced by the control implant, not actual adipose tissue accumulation.

Interestingly, it was recently reported that bone marrow mononuclear cells used in tissue engineered vascular grafts had disappeared from the grafts within a few days (Roh et al. 2010; Hibino et al. 2011). The newly formed vessels in tissue were then found to be solely derived from the host, and the cells in the graft actually just promoted the host tissue regeneration instead of themselves differentiating into the vessels (Hibino et al. 2011). A significant proportion of the beneficial effects of cell therapy have also previously reported to be from the angiogenic factors secreted by the cells (Rehman et al. 2004). These studies support our hypothesis and current results that efficient acellular inductive angiogenesis/adipogenesis is possible by using solely an appropriate combination of natural cytokines, eluting from the scaffold into tissue (Roh et al. 2010; Hibino et al. 2011).

Donor and species variability of adipose tissue extract

The cytokine profiles of different ATE lots revealed slight variations between donors. There were also donor differences in adipogenesis induction. All ATE lots induced adipogenesis, nevertheless, some lots were slightly more potent than others. The observed differences between donors may reflect differences in the BMIs of the individuals, values which were not systematically followed during the study. The donor age seemed not to have effect on the adipogenic potential. The possible effect of gender could not be determined, as most of the donors were females. The lot differences of ATE were detected also in angiogenic potential, but angiogenesis induction was also composition (time-point) dependent. The found differences may also be due to the preparation process of ATE. The processing of very different sizes of tissue samples and changes in the extraction volumes contributed to the variation in protein and cytokine concentrations.

ATE had also species variability in the protein concentration and in the *in vivo* effectivity. Human ATE induced more capillary formation *in vivo* than rat ATE in rat subcutaneous tissue. Higher protein concentration of human ATE compared to rat ATE may have had contribution to this. Nevertheless, as rat ATE was overall slightly more efficient in adipose tissue accumulation in rat tissue than human ATE, the results could also reflect the marked known differences between rodent and human adipose tissue in the expression, response and function of adipokines (Wang et al. 2008). As human (xenogenic) factors seem to act differently in rat adipose tissue remodeling than rat (allogenic) factors, these possible species differences set challenges to the further *in vivo* studies with the extract.

Adipose tissue extract, a novel acellular inductive agent, is potential for clinical use

The adipose stem cell therapy is currently the most common soft tissue engineering strategy. Although promising results have been achieved with this therapy approach, it is time-consuming as cells need to be expanded *in vitro*. Moreover, in this approach, more than one operation is required, and only autologous therapy is possible. In the current study, we aimed at producing a cell-free, protein-rich extract with an easy and gentle extraction method. As this procedure does not require any special equipment or refined conditions, it could be carried out e.g. in operation room conditions during surgery. The protein and growth factor concentrations of several extracts from different extraction conditions were measured during this study. The extraction times varied from 15 min to 48 hours, and several aliquots were also collected from single sample. Two different incubation conditions were also tested (room temperature

or 5% CO₂ buffered 37°C cell incubator. In all of the conditions, protein and growth factor concentrations were similar. Even when short time points were used, efficient protein production was achieved. Interestingly, in a clinical point of view, the sample taking, extraction and applying the extract into patient wound or defect would be possible during single surgery from one small tissue sample. This could reduce markedly costs and support patient comfort and recovery.

Chronic wounds are an enormous current medical challenge. It has been reported, that the costs of chronic leg ulcer treatment could be reduced by a careful selection of the dressing product (Muller-Buhl et al. 2012). In addition, in studies in Sweden and UK, the frequency and duration of dressing changes was shown to have substantial effect on the treatment costs (Ragnarson Tennvall and Hjelmgren 2005). ATE could be potentially used to induce also chronic wound healing. The use of ATE, if successful, would progress healing, or, importantly, reduce the frequency of needed dressing changes. This could markedly reduce the cost of chronic wounds.

During *in vivo* studies, no adverse reactions or fibrous capsule formation occurred. The evaluation of inflammatory effects in tissue revealed that ATE-implants did not show increase in irritating effects over the control implant. However, mild inflammatory cell infiltration was seen shortly after implantation. Macrophage infiltration is known to be a key inducer of adipose tissue remodeling (Rhodes 2007; Saillan-Barreau et al. 2003; Sun et al. 2011). Moreover, macrophages are known to promote angiogenesis (Ye and Gimble 2011) and to be necessary for neoformation of vessels in host tissue (Hibino et al. 2011). A mild inflammatory response, also seen in the current study, is therefore known to be beneficial and according to Breuer (2011), actually essential for the tissue regeneration. Moreover, there was no difference in the immunological reactions between allogenic (rat ATE in rat) and xenogenic (human ATE in rat) implants. A key feature of a soft tissue substitute, good biocompatibility (Choi et al. 2010) was therefore achieved with the ATE-HA implant; thus, ATE can be considered to be biocompatible and safe and has potential to be used clinically.

However, there are current reports on the negative side-effects of the clinical use of inductive growth factors. Possible side-effects of angiogenic growth factor therapy can be hemangioma, hypertrophic scar or glomeruloid body formation, and systemic effects tumor formation, retinopathy or arthritis (Beer et al. 1998; Dor et al. 2003; Markkanen et al. 2005; McHoney 2010). A warning example is Regranex (becaplermin), a recombinant PDGF-B protein product approved for clinical use for the treatment of chronic wounds. Regranex gel contains 100 µg of protein per 1g of gel, and is commonly administered daily. In long term safety studies this product users have been reported to have increased cancer risk. (EMA, 2010) Moreover, individuals treated with recombinant human therapeutic proteins have been reported to develop autoantibodies against cytokines and growth factors such as to interferons, interleukins, GM-CSF and TNF

(Arend et al. 1991; Meager et al. 2010). However, the biological significance of these auto-antibodies is unclear as they may be associated with pathological conditions, but they are also suggested just to be part of normal mechanism preventing growth factor overstimulation (Sauerborn et al. 2011; van der Meide and Schellekens 1997). Due to the reported side-effects, the use of high concentrations of inductive growth factors should be avoided (Brey et al. 2005; Epstein et al. 2001). Although these safety precautions may not be underestimated, ATE is suspected to be safer than currently used growth factor therapies. As ATE has the natural combination of a variety of factors, the overall doses of adipogenic and angiogenic factors are markedly reduced from single growth factor therapies. Compared to e.g. Regranex, the concentrations of growth factors are 100 to 1000 times lower, and when combined to a biomaterial, the concentration and activity of the extract can be controlled. Moreover, in animal tests, only one fairly low dose was found to be inductive, so the administration schedule would be most likely less frequent than with current methods. Finally, ATE is not a recombinant protein product, but it is completely natural, and the first clinical studies would be performed with autologous extract.

Development of human in vitro vascular analogues

From July 11, 2013 onwards, the European Union directive prohibits the performance of animal testing in the European Union for cosmetic products or ingredients of cosmetic products (EC 2009). As the currently used (angiogenesis) *in vitro* methods have wide variability and they often lack *in vivo* relevance (Auerbach et al. 2003), there is an urgent need for appropriately validated *in vitro* assays that would provide relevant results of the effects of drugs and chemicals in human. Human cell and tissue models represent a more reliable way of predicting the effectiveness of drugs in humans, and moreover, adequate *in vitro* assays would reduce the use of animal tests in preclinical screening studies.

hASC contribute to angiogenesis and have multipotentiality in hASC angiogenesis in vitro assays

The recruitment of pericytes along vascular tubules is an essential step in vessel maturation, needed to induce basement membrane formation and to prevent vessel regression (Gerhardt and Betsholtz 2003). The failure of endothelial cells to establish mature structures *in vitro* is often due to absence of stabilizing mural cells (Merfeld-Clauss et al. 2010). Moreover, the creation of 3D tissue models and di- or tri-culture of cells may be challenging. Several different solutions e.g. fibroblast feeder layers, coating

of wells or 3D matrices with natural ECM components, as well as inductive growth factors have been tested while trying to improve the survival and induction of the cells. However, scaffolds, often used to create multilayered tissue constructs, may also interfere with the cell-to-cell interactions and the cell assembly (Norotte et al. 2009).

In the current study, our specific effort was to develop an inductive prevascular-like network with properties of mature vessels that could be used for studying angiogenesis *in vitro*, and especially, that would aid in the development of tissue models by acting as a natural scaffold and supporting target cell differentiation. The specific emphasis was on use of unsorted ASC. As ASC have strong plasticity, high proliferation and differentiation capacity and they provide angiogenic environment by secreting inductive factors, they are an ideal component for *in vitro* angiogenesis modeling. Unsorted stromal cells have been previously shown to promote angiogenesis and myogenesis *in vivo* (Iwashima et al. 2009; Kang et al. 2010). Moreover, co-culture of endothelial cells and mesenchymal cells has been previously shown to promote formation of capillary network *in vitro* and *in vivo* (Koike et al. 2004; Merfeld-Clauss et al. 2010; Kang et al. 2009). However, only few studies have previously characterized human hASC and HUVEC scaffold-free co-cultures and their angiogenic properties (Merfeld-Clauss et al. 2010; Traktuev et al. 2008) or hASC monoculture angiogenic properties (Miranville et al. 2004).

hASC and HUVEC angiogenesis assay had excellent reproducibility and minimal limitations in technical performance. Although the proliferative and differentiation capacity of adult stem cells is known to decrease after serial passaging (Hass et al. 2011; Lindroos et al. 2009), hASC and HUVEC co-culture was passage independent. The growth factor induction and co-culture induced high proliferation of both cell types in hASC+HUVEC co-culture, which may explain the passage independency. hASC did not only stimulate the tubule formation and maturation, but were also seen to differentiate into endothelial-like cells in this system. This was seen as a mosaic of GFP-infected HUVEC and hASC in angiogenesis assay. Moreover, in hASC monoculture, PECAM-1 gene expression was increased indicating endothelial differentiation, which was also seen as vWf-positive tubules in hASC monoculture. Although freshly isolated hASC, or more precisely, stromal-vascular fraction cells, may contain microvascular endothelial cells (Rehman et al. 2004; Wosnitza et al. 2007), hASC identical to this study have been shown to lack the expression of hematopoietic and angiogenic markers e.g. CD31, CD106 and CD146 (surface marker expression 0.6 ± 0.7 , 0.7 ± 0.7 and 0.4 ± 0.2 , respectively (Lindroos et al. 2009; Lindroos et al., 2010). This study therefore supports the earlier studies (Planat-Benard et al. 2004; Rubina et al. 2009; Traktuev et al. 2008) and demonstrates that hASC are able to contribute to vessel formation maturation *in vitro* by stimulating endothelial cell proliferation and also by differentiating into mature vascular structures with endothelial, pericytic and smooth muscle cell properties. Moreover, the

basement membrane formation in this extent has not been shown in any of the previous *in vitro* studies. Most of the current angiogenesis assays do not express basement membrane markers and therefore can be suspected to consist of immature tubules with no essential elements of mature tubules.

The tissue engineered grafts are limited in size due to a lack of proper induction of vascularization (Jain et al. 2005; Norotte et al. 2009). hASC and HUVEC angiogenesis tubule network assay could be particularly suitable for engineering different tissue models. The hASC and HUVEC angiogenesis assay presents an advanced feature, a three-dimensional multilayered tubule network, with thickness of around 200 μm . This tubule network could allow the creation of multilayered, yet, scaffold-free, tissue constructs, especially for soft tissue applications. This dynamic structure might provide the missing inductive platform for 3D tissue models, the major issue that has not yet been resolved for *in vitro* tissue models or implantable constructs.

To accelerate the use of this angiogenesis assay in tissue engineered constructs, we developed a xeno-free modification for the assay. From a clinical point of view, to use this tubule network in implantable constructs, animal derived components such as fetal serum should essentially be removed. Moreover, the cells used in the constructs must be tissue- and donor-specific. Currently, HUVEC are used as endothelial cells in the assay, as they are a widely used cell source, easy to obtain, carefully characterized in our laboratory and have excellent proliferation capacity *in vitro*. However, as endothelial cells are known have specialized phenotypes depending on the tissue (Jain 2005), tissue –specific endothelial cells are most likely required for tissue grafts. However, endothelial cells are also known for their high plasticity and the ability to respond to their microenvironment (Garcia-Cardena et al. 2001). According to the study by Merfeld-Clauss et al. (Merfeld-Clauss et al. 2010), the endothelial cells could be possibly replaced with any tissue specific endothelial cells (e.g. microvascular endothelial cells in adipose tissue) with no effect on tubule formation capability in this type of *in vitro* assay. Therefore, we can suspect that this developed tubular network platform is easily modified to be suitable in several different *in vitro* tissue model applications where dense tubular network is needed. However, for implantable constructs, the comparison of different endothelial cell sources must be performed.

Adipose stromal cells, on their behalf, have less immunogenicity and their potential to be used for allogeneous therapy is currently under prominent research. For this reason, the hASC monoculture assay here studied would be especially fascinating for tissue engineering. The angiogenic differentiation potential of hASC monoculture was presented in this study. In addition to the current study, the tubule formation in plain hASC scaffold-free monoculture has been shown previously only by Miranville et al. (Miranville et al. 2004) and Heydarkhan-Hagvall et al. (Heydarkhan-Hagvall et al. 2008). Nevertheless, the hASC monoculture

angiogenesis assay requires further optimization of the hASC culture conditions, such as cell number, the composition of inductive factors and cell passage, as the hASC monoculture angiogenesis assay did not show adequate repeatability to differentiate towards capillaries. There were outstanding differences between donors, which is largely due to the heterogeneous nature of SVF cells.

Intra-laboratory validated in vitro angiogenesis assay is a relevant bioassay in predicting effects of chemicals in humans

In intra-laboratory validation, we standardized an angiogenesis *in vitro* assay under GLP quality system, and studied how well the assay reflects the current *in vivo* results.

Optimization of the critical steps and factors in this assay, especially HUVEC isolation, passage and master bank were crucial for the outcome and the adequate performance of the assay. Due to the biological nature of the test, slight variation in positive control value was observed, and therefore we were obliged to allow a range of values (with minimal average score of 5.75 in the analysis scale) for positive control in the technical quality control criteria. This pre-set acceptance criteria resulted in significant person to person variation between technicians in positive control value during validation. Although a routine method, this assay is a biological system that is very sensitive to even minimal changes in the technical procedure. The variation between technicians did not have effect on the overall outcome of the performance of the assay, as the results were compared to internal positive and negative controls in each testing time in the assay. Interestingly, the inhibitory concentrations from our study were in good concordance with the therapeutic plasma concentrations from clinical studies (maximal concentration in plasma, C_{max}), however, they did not reflect the results of currently used animal tests.

Currently, the applicability domain of this intra-laboratory validated angiogenesis assay contains pharmaceuticals and conditioned media of cells. However, for optimal biological performance, the variable test items may require adjustments in the setup of this assay, like adjustment of test media. The only restrictions of the assay are the solubility and solvent of the test chemicals, as the commonly used solvents (DMSO, ethanol and methanol) have accepted limits in cell culture (approximately maximal 0.5% of the cell culture medium). This may in some cases reduce the concentrations of chemicals that can be tested in the assay.

It is also noteworthy, that in validation, the reproducibility and applicability of the assay is tested and GLP assures the adequate quality of the studies, but neither of these guarantees the biological relevance of the assay. This needs to be studied in optimization phase prior to validation phase. Therefore, in addition to the reference chemicals, the functionality of the assay was earlier tested in the optimization phase with the following

chemicals; endostatin, interleukin-1 soluble receptor 1 (IL-1 SR1), suramin, mevinolin, paclitaxel, fumagillin, anti-EGFR, cyclo-oxygenase-2-specific inhibitor NS-398, indomethacin, VEGF, bFGF, anti-bFGF and PDGF-B.

The overall variation in controls, and variation between different test times was very low (CV%=1.39% and CV=0.34-2.37%, respectively). This angiogenesis assay can be therefore concluded to be successfully intra-laboratory validated and can now be used for drug screening in the preclinical phase of drug discovery. Moreover, as most of the validated *in vitro* assays are used for safety studies, this currently validated *in vitro* angiogenesis assay is one of the few validated routine assays that are used for efficacy studies in addition of being applicable also for testing of angiotoxicity of chemicals.

Future perspectives

The demand for the engineered soft tissue is increasingly growing. Effective cell free constructs would be potent for allogeneous use and allow the development of commercially available "off-the-shelf" products. As ATE was found to be biocompatible, it has potential to become this type of tissue product. However, the results of small animal studies need further verifications until applied to clinical therapy, and therefore large animal studies need to be performed. In addition, the first clinical studies will be performed with autologous material.

In the current *in vivo* study, the administration of the injectable implant was performed only once, at the onset of the study, with fairly low concentrations of ATE, but with effective results. Therefore, the inductive effect of ATE could potentially be even improved with repeated administration or increased dose of ATE. However, the optimal dose and efficacy, and administration schedule, i.e. the therapeutic window for ATE requires further investigation. Moreover, the systemic toxicity and the safety assessment (non-tumorigenicity) of ATE, especially in high concentrations, as well as the effects of simple ATE substance need to be evaluated. The current research for ATE therefore concentrates on improvement of safety of the product as well as on the development of allogeneous product and studying the optimal dosage of ATE.

The possibility to modify the cytokine content of ATE is also a tempting thought. As the angiogenic and adipogenic factors are known to act in a cascade in different phases of the refined process of adipose tissue formation as reviewed e.g. by Papetti and Herman (2002), there is a possibility to stimulate different phases of angiogenesis /adipogenesis by a simple adjustment of the extraction time of ATE, or further tailoring ATE by precipitating single or a few cytokines from the extract with presently available methods. For instance, as IL-6 is known to e.g. impair blood-brain-barrier function by increasing the permeability of endothelium

(Abbott et al. 2006), the presence of high IL-6 in ATE, in certain cases, might not be preferable. This could be avoided by simply using the 1hr time point extract, or by precipitating IL-6 from the 24 h extract. However, the optimal composition of ATE for different applications needs further studies. It also remains to be resolved whether the range of indications of ATE could be broadened i.e. whether the inductive effect of ATE applies to other tissues than soft tissue.

Interestingly, proresolving lipid mediators, resolvins, synthesized from omega-3 fatty acids, are known to suppress inflammation, and deficiency of these mediators leads to delayed wound healing and inflammation in diabetes (Hellmann et al. 2012). As an adipose tissue product, ATE also contains lipids and could therefore well be a source of these wound healing inducers. This would be an additional advantage of ATE. However, prior to further conclusions in this task, the precise triglyceride content of ATE needs to be further characterized.

No *in vitro* assay of natural adipogenesis yet exists (Bucky and Percec 2008; De Gemmis et al. 2006). However, it is important to obtain information on expression and functional role of pro-and anti-angiogenic components as well as adipogenic agents in *in vitro* models of adipogenesis (Christiaens and Lijnen 2010). As ATE was found to be efficient for modeling natural adipogenesis *in vitro*, ATE has potential to be used in the development of a standardized adipogenesis cell assay for preclinical drug and chemical screening. For *in vitro* use, the donor-dependent variation seen in our study, is possible to overcome by using a pool of extracts from several donors. ATE may also be used as a specific cell culture supplement in different *in vitro* studies.

The applicability domain of the intra-laboratory validated angiogenesis assay will be possibly further expanded in future studies by testing the assay with different chemicals. Moreover, the possibility of the use of hASC and HUVEC angiogenesis assay in drug and chemical screening will be explored.

An intriguing application for future studies of tissue engineering and 3D *in vitro* tissue models would be the combination of ATE and hASC tubule network structure. It has been reported that immature vessels are mostly regressed *in vivo* and are not integrated into host tissue after implantation (Rouwkema et al. 2006). However, if we combined ATE with the tubules, we could possibly enhance the blood vessel maturation and integration into host tissue and even further, create a unique 3D soft tissue model.

Summary and conclusions

In this study, a novel cell-free angiogenesis and adipogenesis inducing agent, adipose tissue extract (ATE) was produced from mature human adipose tissue. ATE contains a wide variety of promoters of angiogenesis and adipogenesis and induces angiogenesis and adipogenesis *in vitro* and *in vivo*. The second part of the study focused on developing *in vitro* methods for angiogenesis induction. hASC angiogenesis assays were developed and fibroblast and HUVEC angiogenesis co-culture assay was validated.

Based on the studies performed, the main findings and conclusions are:

- ATE contains all the most important angiogenic and adipogenic factors of mature adipose tissue.
- ATE induces adipogenesis and angiogenesis *in vitro* in respective cell culture assays.
- ATE is able to induce efficient soft tissue restoration with normal vascular remodeling. The effect is sustained, and remains after scaffold degradation.
- ATE-implants do not show increase in irritating effects over the control implant. No adverse reactions or fibrous capsule formation occur, and therefore ATE can be considered to be biocompatible and safe and applicable for clinical use.
- The current study supports the previous results that the tissue engineering grafts need not to contain cells. The natural inductive cytokines, in an appropriate combination, eluting from the scaffold into tissue, can be sufficient for the stimulating angiogenic/adipogenic effect.
- ATE was found to be efficient for modeling natural adipogenesis *in vitro*, and therefore ATE has potential to be used in the development of a standardized adipogenesis routine cell assay to be used in preclinical phase of drug discovery for efficacy testing of novel drugs for e.g. obesity and diabetes.
- Through optimization and intra-laboratory validation, we obtained a routine angiogenesis assay with minimal variation (overall CV=1.39%). This human cell *in vitro* angiogenesis assay mimics the *in vivo* effects, and thus it can be used to supplement animal tests in efficacy and angiotoxicity testing of pharmaceuticals and chemicals.

- The adipose stromal cell 3D multilayered mature tubule network is an excellent tool for studying cell interactions during vascular development, and moreover, is useful for designing multilayered, scaffold-free, *in vitro* tissue models or implantable constructs.

It can be concluded that ATE is an angiogenic and adipogenic substance that is also capable of inducing cell proliferation and activating further cytokine production in tissue. ATE has potential to be used to induce revascularization of ischemic tissues and to be used in tissue products that fail due to inadequate vasculature in soft tissue engineering. The developed *in vitro* angiogenesis assays are valuable tools for basic research and preclinical drug screening studies as well as for tissue engineering applications.

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Original publications

Human Adipose Tissue Extract Induces Angiogenesis and Adipogenesis *In Vitro*

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The induction of adequate vascularization, a major challenge in tissue engineering, has been tried with numerous methods but with unsatisfactory results. Adipose tissue, an active endocrine organ with dense vasculature, secretes a wide number of angiogenic and adipogenic factors and seems an attractive source for these bioactive factors. We produced a novel cell-free extract from mature human adipose tissue (adipose tissue extract [ATE]) and analyzed the ability of this extract to induce angiogenesis and adipogenesis *in vitro* and studied the cytokine and growth factor composition of ATE with ELISA and cytokine array. We demonstrate that ATE, when added as cell culture supplement, effectively induced triglyceride accumulation in human adipose stem cells at concentrations from 200 µg/mL upward in less than a week and caused elevated levels of adipocyte differentiation markers (proliferator-activated receptor gamma and acyl-CoA-binding protein) when treated with at least 350 µg/mL of ATE. ATE induced angiogenesis from 450 µg/mL upward after a week *in vitro*. ATE contained numerous angiogenic and adipogenic factors, for example, vascular endothelial growth factor, basic fibroblast growth factor, interleukin-6, adiponectin, angiogenin, leptin, and insulin-like growth factor-I, as well as lower levels of a wide variety of other cytokines. We here present a novel cell-free angiogenesis- and adipogenesis-inducing agent that is cell-free and easy to produce, and its effect is dose dependent and its composition can be easily modified. Therefore, ATE is a promising novel agent to be used for angiogenesis induction to overcome the challenge of vascularization and for adipogenesis induction in a wide variety of tissue engineering applications *in vitro* and *in vivo*. ATE is also efficient for reproduction and modeling of natural adipogenesis *in vitro* for, for example, obesity and diabetes studies.

Introduction

NUTRIENT SHORTAGE DUE to inadequate vascularization limits the size of tissue-engineered implants¹⁻⁴ and prevents their successful use in tissue engineering.³⁻⁵ Glycosaminoglycans, single synthetic growth factors (e.g., vascular endothelial growth factor [VEGF] or basic fibroblast growth factor [bFGF]), or *in vitro* differentiated cells have been used for angiogenesis induction,^{1,6-8} but the results have been only modest and the obtained effect transient.⁹ To gain sustained and significant induction of angiogenesis, simultaneous effect of multiple growth factors is needed.^{1,10,11} However, the use of synthetic growth factors is expensive, and moreover, tailoring the optimal combination of growth factors has proven to be extremely difficult.¹²

Adipose tissue is an active endocrine organ consisting of mature adipocytes, adipose stem cells, macrophages, endo-

thelial cells, and extracellular matrix components.^{13,14} Adipose tissue is rich in blood vessels and a major source of growth and differentiation promoting factors (cytokines and adipokines)^{10,15-20} known to release bioactive levels of, for example, leptin, adiponectin (Acrp30), angiotensin II, acylation-stimulating protein, adipisin, resistin, prostaglandins, glucocorticoids, cytokines (for example, tumor necrosis factor- α [TNF- α], interleukins [IL] 1 β , 6, 8, and 10), chemokines (for example, CC-chemokine ligand 5 [CCL5] and monocyte chemoattractant protein [MCP-1]), growth factors (for example, hepatocyte growth factor [HGF], VEGF, bFGF, insulin-like growth factor [IGF], and transforming growth factor β [TGF- β]), angiopoietins 1 and 2 (Ang-1 and Ang-2, respectively), and plasminogen activator inhibitor 1.^{2,10,20-26} Several adipose tissue-derived growth factors and cytokines stimulate angiogenesis and the formation of an extensive capillary network required for the expansion and optimal

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function of the adipose tissue.^{25,27–29} The endocrine functions and secreted factors as well as angiogenesis of adipose tissue are reviewed in detail by Galic *et al.*,³⁰ Poulos *et al.*,¹³ Karastergiou and Mohamed-Ali,¹⁴ Kershaw and Flier,¹⁷ and Christiaens and Lijnen.³¹ The advance of the use of adipose tissue is that it is abundant and easy to obtain with minimal invasive methods.^{20,32} Further, the methods for adipose stem cell isolation and differentiation are well described in literature.^{33–35}

In the light of the present knowledge of adipose tissue function, we explored a new way of producing growth- and differentiation-promoting factors of human origin. We extracted the factors from mature adipose tissue without affecting the cell viability and studied the composition and angiogenic and adipogenic potential of this adipose tissue-derived cell-free extract (adipose tissue extract [ATE]). We characterized ATE protein and cytokine content and studied the effect of ATE on angiogenesis and adipogenesis at different concentrations *in vitro*. Three important factors were characterized quantitatively with ELISA and a large variety of factors qualitatively and semiquantitatively with cytokine array. We here describe a novel method for producing growth- and differentiation-promoting factors from human adipose tissue and the detailed composition of the extract as well as the ability of the extract to induce angiogenesis and adipogenesis. We demonstrate that this protein-rich extract has strong angiogenic and adipogenic potential and could be used in angiogenesis or adipogenesis induction in a wide variety of tissue engineering applications *in vitro* and *in vivo* to overcome the major challenge of induction of vascularization.

Materials and Methods

The study was conducted in accordance with the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland (R03058, R08028). The adipose tissue samples were obtained from surgical operations and the human umbilical cords from scheduled caesarean sections with informed consents at the Tampere University Hospital, Tampere, Finland.

ATE preparation

Adipose tissue specimens were cut into small pieces and transferred into 50 mL tubes (Sarstedt). Equal volume of phosphate-buffered saline (PBS) or, for cell culture studies, Dulbecco's modified Eagle's medium Nutrient Mixture F-12 (DMEM/F12; Gibco, Invitrogen) without supplements were added into the tube and left for a minimum of 15 min for growth factor secretion at room temperature or at 37°C in a CO₂ incubator. The tube was gently shaken during incubation. The extracts were collected at predetermined time points (15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, or 24 h), centrifuged at 12,000 rpm for 5 min, and sterile-filtered through 0.2 µm filter (Sarstedt). The aliquots were stored at –20°C until use.

Protein and growth factor expression of ATE

ATE samples derived from different patients and/or collected at several different time points (described earlier) were analyzed for the total protein concentration with BCA pro-

tein Assay Kit (Pierce Biotechnology) at different aliquots. The total protein concentration was measured from all of the tested time points (15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, or 24 h). The concentrations of VEGF, bFGF, and IGF-I were measured from a large number of human ATE samples from two different time points (1 and 24 h) by enzyme-linked immunosorbent assay (ELISA; Quantikine Human VEGF Immunoassay, Quantikine Human FGF basic Immunoassay, or Quantikine Human IGF-I Immunoassay, respectively; R&D Systems) according to the manufacturer's instructions. All standards and samples were measured as duplicates.

Cytokine array analysis

Altogether, 120 growth factors and cytokines were tested from six different extract samples at two different time points (1 and 24 h) with RayBio® Human Cytokine Antibody Array C Series 1000 (RayBioTech, Inc.). The array was performed according to the manufacturer's instructions. The chemiluminescence was detected with FluorChem™ 8800 imaging system (Alpha Innotech). The chemiluminescent signals were measured with FluorChem software v. 3.1. and analyzed qualitatively and semiquantitatively.

Isolation and culture of human adipose stem cells

Stem cell isolation procedure was performed as previously described.^{33,35,36} Briefly, human adipose tissue specimens were cut into small pieces and enzymatically digested with 0.05% collagenase I (Invitrogen) in DMEM/F12 for 60 min at 37°C in a gyratory water bath. The digested tissue was centrifuged at 600 g for 10 min at room temperature. The digested tissue was filtered through a 100 µm filter (Sarstedt), centrifuged, and filtered through a 40 µm filter (Sarstedt). Human adipose stem cells (hASCs) were seeded in DMEM/F12 supplemented with 1% L-glutamine (L-glut; Gibco), 1% antibiotic–antimycotic mixture (AB/AM; Gibco), and 15% human serum (HS; Cambrex) into 75 cm² cell culture flasks ((Nunc EasyFlask™; Nunc) and allowed to attach overnight. The next day, the cells were washed several times with PBS, and the medium was changed to remove the debris. The hASCs were maintained at 37°C under a 5% CO₂ air atmosphere at a constant humidity. When to the cells reached 70% confluence, they were divided at a ratio of 1:2–1:3 or further used for adipogenesis studies.

Adipogenesis induction

To initiate adipogenic differentiation, hASCs were plated at early passages (p1–p5) at a density of 10,000 cells/cm². The next day, six different culture conditions were applied to hASCs. Cells were cultured in (1) the hASC culture medium: DMEM/F12, 1 mM L-glut, 1% AB/AM, and 15% HS; in (2) the adipogenic control medium: DMEM/F12, 10% fetal bovine serum (FBS), 1% AB/AM, 1% L-glut, 33 µM biotin (Sigma Aldrich), 17 µM pantothenate (Sigma), 100 nM insulin (Sigma), 1 µM dexamethasone (Sigma), and 0.25 mM isobutyl-methylxanthine (IBMX; Sigma; IBMX was left in the adipogenic culture medium for the first 24 h only); in (3) DMEM/F12, supplemented with 15% HS, 1% penicillin/streptomycin (Gibco), 1 mM L-glut, and 350 µg/mL of ATE; in (4) DMEM/F12, supplemented with 15% HS, 1% penicillin/streptomycin, 1 mM L-glut, and 700 µg/mL of ATE; in (5)

DMEM/F12, supplemented with 15% HS, 1% penicillin/streptomycin, 1 mM L-glut, and 950 µg/mL of ATE; and in (6) DMEM/F12, supplemented with 15% HS, 1% penicillin/streptomycin, 1 mM L-glut, and 1200 µg/mL of ATE. The medium was changed every 3 days. Cells were cultured for 1 week, 2 weeks, or 4 weeks and examined for lipid accumulation by Oil-Red-O (ORO; Sigma) staining or further used for quantitative reverse transcriptase-polymerase chain reaction (RT-PCR).

ORO staining

Lipid accumulation was assessed at 1 and 4 weeks after the onset of the differentiation experiment by using ORO staining. hASCs were fixed with 4% paraformaldehyde (Sigma) for 20 min and rinsed several times with PBS. ORO stock (0.5%) solution was prepared in 100% isopropanol (Merck & Co., Inc.), and for staining, it was diluted 3:2 in distilled water, left at room temperature for 10 min, and filtered through standard filter paper. hASCs were incubated with 60% isopropanol for 2–5 min, incubated with the ORO staining solution for 5 min, and rinsed several times with PBS. Phase-contrast micrographs of the stained cells were taken with Nikon Eclipse TS-100 microscope (Nikon) equipped with Nikon DS Camera Control Unit DS L-1 (Nikon). The images were processed with Adobe Photoshop software 7.0 (Adobe Systems) and Corel Draw software 10.0 (Corel Corporation). The extent of ATE-induced triglyceride accumulation was evaluated with ORO extraction at 1 week from hASCs cultured with 1 mg/mL of ATE. The PBS was removed from wells and the wells were allowed to dry. ORO was extracted from the cells by 10 min incubation with 100% isopropanol. The absorbance of the extracted ORO from hASCs was measured at 510 nm with Victor3 1420 Multilabel Counter (Perkin Elmer).

Quantitative RT-PCR

Primers used for adipogenesis-specific genes were *acyl-CoA-binding protein (ACBP)*, a protein increased in cells with high turnover of fatty acids,³⁷ *peroxisome proliferator-activated receptor gamma (PPARγ)*, a key inducer of adipogenesis and in part responsible for the induction of ACBP during adipocyte differentiation,³⁷ and a reference house keeping gene *ribosomal protein large P0 (RPLP0)*. All oligonucleotides were from Oligomer Oy and are shown in Table 1. The total RNA was extracted from 7 or 28 days cultured hASC using TRIzol® (Invitrogen) following the manufacturer's protocol. cDNA was synthesized using High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems) according to manufacturer's instructions. Quantitative RT-PCR analysis was performed in a 96-well optical reaction

plate with an ABI Prism 7000 sequence detector (Applied Biosystems Inc). Reactions were performed using SYBR Green PCR Master Mix kit (Applied Biosystems), 50 ng cDNA sample, and 10 µM primers using conditions of 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 60 s at 60°C. All reactions were performed in duplicates. The results were processed with ABI Prism 7000 PCR Quantification Software (Applied Biosystems). Relative RNA expression was calculated in comparison to RPLP0 RNA expression using the method by Pfaffl³⁸:

$$\text{Ratio of relative expression} = \frac{(E_{\text{target}})^{\Delta C_P \text{ target (control sample)}}}{(E_{\text{ref}})^{\Delta C_P \text{ ref. (control sample)}}}$$

Coculture of human umbilical vein endothelial cells and fibroblasts

The human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins as described by Jaffe *et al.*³⁹ Briefly, the cord was separated from the placenta, the umbilical vein was cannulated with a 20G needle, and the needle was secured by clamping the cord over the needle with a surgical clamp. The vein was perfused with PBS to wash out blood and then the opposing end of the umbilical vein was clamped with a surgical clamp. Subsequently, the vein was infused with 0.05% collagenase I. The umbilical cord was incubated in a cell incubator at 37°C for 15 min. After incubation, the collagenase I solution containing HUVECs was flushed from the cord by perfusion with PBS into a 50 mL polypropylene tube (Sarstedt). The cells were centrifuged at 200 g for 10 min, washed once with medium, centrifuged again, resuspended in EGM-2 BulletKit medium (Lonza), and seeded into 75 cm² flasks. Medium was changed every 3 days.

Human foreskin fibroblasts, BJ cell line, were purchased from American Type Culture Collection (CRL-2522; ATCC) and cultured in minimal essential medium (Gibco) supplemented with 10% FBS, 1% L-glut, 1% nonessential amino acids (Gibco), and 1% AB/AM. To study the angiogenic potential of the ATE, an angiogenesis coculture assay was established as previously described by Friis *et al.*⁴⁰ and Saranen *et al.*⁴¹ Briefly, fibroblasts were seeded at a density of 20,000 cells/cm² into 48-well plate and were grown to confluence (3 days). Next, the HUVECs were seeded on top of confluent fibroblast cultures at a density of 4000 cells/cm² in EGM-2 BulletKit medium and were allowed to attach overnight before angiogenesis induction.

Angiogenesis induction

Five different treatments were applied to cells the day after plating HUVECs on top of the BJ fibroblasts (described earlier). Treatments were (all in duplicates) (1) negative control: the endothelial cell basal medium (EBM-2; Lonza) supplemented with 0.1% gentamicin (GA-1000 from EGM-2 BulletKit), 2% FBS, and 1% L-glut; (2) positive control: EBM-2 supplemented with 2% FBS, 0.1% GA-1000, and 1% L-glut, as well as 10 ng/mL VEGF (R&D Systems) and 1 ng/mL bFGF (Sigma); (3) EBM-2 supplemented with 2% FBS, 1% L-glut, 0.1% GA-1000, and 450 µg/mL of ATE; (4) EBM-2 supplemented with 2% FBS, 1% L-glut, 0.1% GA-1000, and 900 µg/mL of ATE; (5) EBM-2 supplemented with 2% FBS, 1% L-glut, 0.1% GA-1000, and 1300 µg/mL of ATE. The media

TABLE 1. OLIGONUCLEOTIDE PRIMERS USED FOR QUANTITATIVE REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION EXPERIMENTS

Antigen	Sequence (5'–3')
RPLP0	F-AATCTCCAGGGGACCAT R-CGCTGGCTCCCACCTTGT
ACBP	F-GCAACTGTGGGCGACATAAA R-GGCCCTTGCCCGTGAAGT
PPARγ2	F-CAGTGTGAATTACAGCAAACC R-ACAGTGTATCAGTGAAGGAAT

were changed once during the culture. The cells were cultured for 7 days before the immunocytochemical staining with an endothelial cell-specific antibody.

Immunocytochemistry

The endothelial tubules in angiogenesis coculture assay were visualized with von Willebrand factor antibody (monoclonal anti-vWF produced in rabbit; Sigma) immunofluorescence staining. Cells were washed three times with PBS, fixed with ice-cold 70% ethanol for 20 min, permeabilized with 0.5% Triton X-100 (JT Baker) for 15 min, and blocked for unspecific staining with 10% bovine serum albumin (Sigma) for 30 min. After blocking, cells were incubated with primary antibody (anti-vWF, 1:500) at 4°C overnight. The following day, cells were washed three times with PBS, incubated 30 min with secondary antibody (polyclonal Antibody to Rabbit IgG FITC; Acris Antibodies GmbH; 1:500), and washed again three times with PBS. After staining, 500 μ L of PBS was left in the cell culture wells, and plates were stored at 4°C. Fluorescence was visualized with Nikon Eclipse TS100 microscope equipped with Nikon DS Camera Control Unit DS L-1, and images were processed with Adobe Photoshop software and Corel Draw software 10.0.

Statistical analyses

All statistical analyses were performed and graphs were processed with GraphPadPrism 5.0 (GraphPad Software, Inc.). Protein concentrations from different ATE aliquots were subjected to one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test and growth factor concentration measurements to nonparametric Mann-Whitney test. RT-PCR results were subjected to one-way ANOVA with Tukey's multiple comparisons test. The variation in ATE-induced adipogenesis was analyzed with unpaired *t*-test. Differences were considered significant when $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

Protein and growth factor expression of ATE

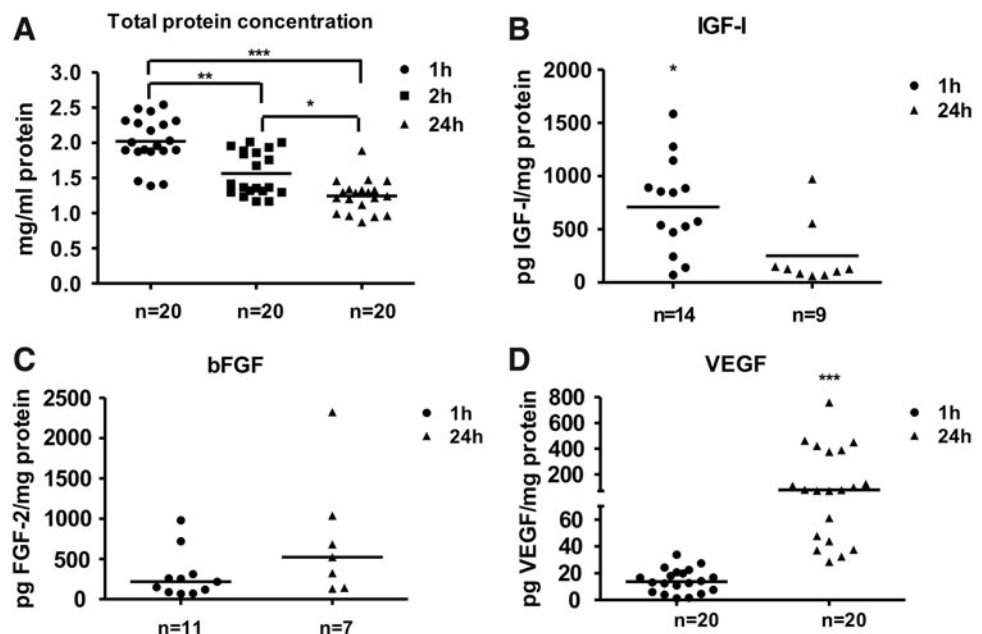
The total protein concentration was measured with BCA Protein Assay Kit (Fig. 1A). The protein concentration was generally highest at 1 and 2 h, after which the protein concentration decreased with time. From the tested time points, three time points were chosen for future use. The selected total protein concentrations (determined with calculated mean) were 2.017 mg/mL (standard deviation [SD]=0.5222) at 1 h, 1.783 mg/mL (SD=0.1516) at 2 h, and 1.120 mg/mL (SD=0.1917) at 24 h. Extracts that were incubated in PBS or in cell culture medium (DMEM/F12) or incubated at room temperature or at 37°C in a cell culture incubator had similar protein concentrations.

The concentrations of IGF-I (Fig. 1B), bFGF (Fig. 1C), or VEGF (Fig. 1D) were measured with ELISA. The IGF-I concentration was significantly higher at 1 h than at 24 h (1 h median=709.6; 24 h median=126.2; $p < 0.0148$). The bFGF concentrations did not significantly differ from each other between time points (1 h median=217.0; 24 h median=522.2; $p < 0.1031$). In contrast to other growth factor results, the VEGF concentrations were significantly higher at 24 h than at 1 h (1 h median=6.020, 24 h median=72.36, $p < 0.0001$).

Adipogenic potential of ATE

The adipogenic potential of ATE was tested by culturing hASCs up to 4 weeks with ATE (Fig. 2A). As a negative control, hASC culture medium (cultured for 14 days) and, as a positive control, adipogenic induction medium (cultured for 28 days) were used. hASCs were cultured in the presence of 350, 500, 700, or 1200 μ g/mL of ATE in 15% HS supplemented medium for 14 days. ATE was shown to induce a homogeneous, dose-dependent, adipogenic conversion to a majority of cells in the culture from early days (3) onward *in vitro*. Mild adipogenic effect was seen when at least

FIG. 1. Total protein concentration and selected growth factor concentrations of ATE. Total protein concentrations (A) were measured with BCA protein assay from ATE samples at 1, 2, and 24 h and IGF-I (B), bFGF (C), and VEGF (D) concentrations with ELISA from ATE samples at 1 and 24 h. The median is shown in each graph as a horizontal line. The significance was tested for total protein concentrations with nonparametric Kruskal–Wallis test followed by Dunn's multiple comparison post-test and for single growth factors with Mann–Whitney test ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$). ATE, adipose tissue extract; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; IGF, insulin-like growth factor.



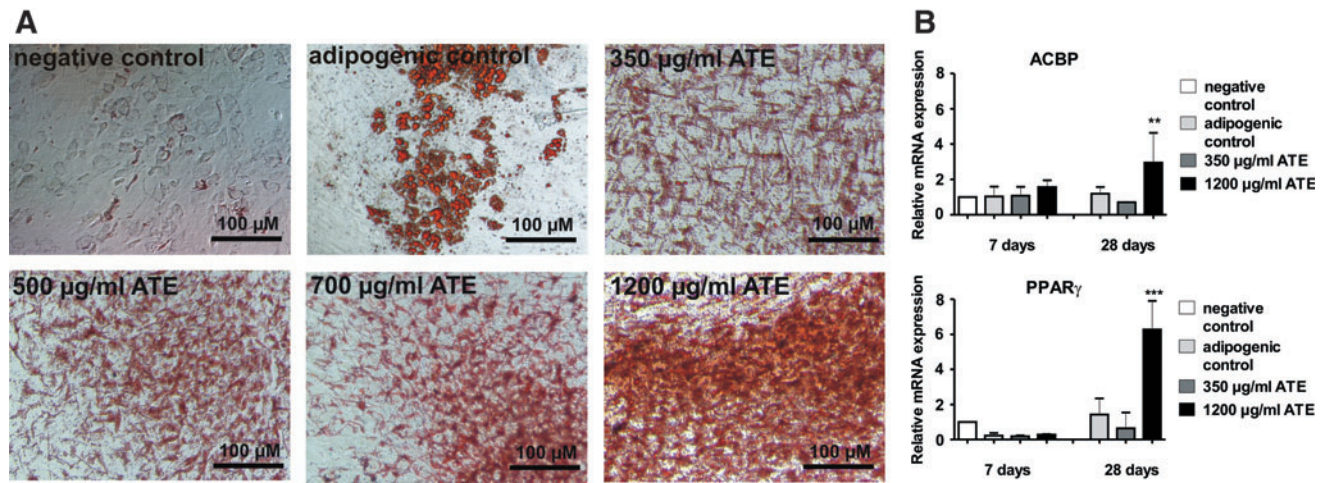


FIG. 2. Adipogenic differentiation of hASCs in the presence of different concentrations of ATE. **(A)** The hASCs were cultured for 14 days, except adipogenic control, which was cultured for 28 days. As a negative control, hASC culture medium was used. Different concentrations of ATE were applied as cell culture supplement causing dose-dependent adipogenic differentiation of hASCs. The cells were stained with Oil-Red-O. Scale bar: 100 μ M in each image. **(B)** The relative mRNA expression levels of PPAR γ and ACBP after 1 or 4 weeks of differentiation. Data are presented as mean \pm standard deviation. The significance was tested with one-way analysis of variance followed by Tukey's multiple comparison test (* p < 0.05, ** p < 0.01, and *** p < 0.001). hASC, human adipose stem cell; PPAR γ , proliferator-activated receptor gamma; ACBP, acyl-CoA-binding protein. Color images available online at www.liebertonline.com/tea

200 μ g/mL of ATE was used (data not shown), the effect being more extensive when longer culture times or higher concentrations of ATE (up to 2000 μ g/mL tested; data not shown) were used. The results represent average of at least 10 separate experiments, each performed as duplicates. The results in Figure 2 are from 1 h extracted ATE containing 1.4 mg/mL protein. The variation in ATE-induced triglyceride accumulation between different adipose tissue samples was quantified with ORO extraction. ATE samples containing 1 mg/mL of ATE (n =32) induced 1.5- to 4.1-fold (median 2.28, SD=0.57, CV=24.7%) triglyceride accumulation compared with adipogenic control treatment. No significant difference in adipogenesis induction (calculated from the same samples) was found between 1 h (n =17) and 24 h (n =15) ATE samples (p =0.878, SD=0.3507, and CV=9.84%).

The results of the relative mRNA expression levels are shown in Figure 2B. The expression of adipogenic markers was dependent on time and dose. One week after the onset of differentiation treatments, only ACBP expression showed mild 1.7-fold induction, when compared with negative control (hASC culture medium). However, at 4 weeks, ACBP expression was increased threefold with high concentrations of ATE (1200–1500 μ g/mL). Similarly, PPAR γ expression was induced almost sevenfold with 1200–1500 μ g/mL of ATE, when compared with controls, and was significantly higher at 4 weeks than at 1 week (p < 0.0001). The adipogenic control medium did not cause elevated expression levels of adipogenesis-related genes in either of the time points.

Angiogenic potential of ATE

The angiogenic potential of ATE was tested by culturing fibroblasts and HUVECs in angiogenesis coculture assay for 7 days with ATE. As a negative control, the HUVECs were grown in normal endothelial cell growth medium. As a positive control (Fig. 3), the HUVECs were treated with

angiogenic growth factors, 10 ng/mL of VEGF, and 1 ng/mL of bFGF. In the presence of ATE, tubule-like structures were developed in the angiogenesis assay from day 4 onward (data not shown). Treatment with ATE stimulated time- and dose-dependent induction of tubule formation in the assay and production of typical long, thin, branched capillary-like structures that were very similar to the angiogenesis positive control, VEGF/bFGF treatment. At day 7, tubule formation was observed in angiogenesis coculture assay treated with 450, 900, or 1300 μ g/mL of ATE. The results represent average of at least 10 separate experiments, each performed as duplicates. The results in Figure 3 are from 1 h extracted ATE containing 1.8 mg/mL protein, 120 pg/mL bFGF, 140 pg/mL IGF-I, and 3.6 pg/mL VEGF.

Cytokine expression pattern of ATE

Altogether, 120 growth factors and cytokines were tested from six different extract samples at two different time points (1 and 24 h). The results of the array are shown in Supplementary Figure S1; Supplementary Data are available online at www.liebertonline.com/tea. Generally, at 24 h, more cytokines were released than at 1 h. All of the highly expressed cytokines at 1 h, angiogenin, adipocyte complement-related protein 30 (Arcp30, adiponectin), IGF-binding protein 3 (IGFBP-3), tissue inhibitor of matrix metalloprotease 2 (TIMP-2), and fibroblast-associated/tumor necrosis factor receptor superfamily member 6, were stably released and were also high at 24 h. However, IL-6, leptin, neutrophil-activating protein 2, CCL5 (e.g., RANTES), Ang-2, bFGF, FGF-9, growth-regulated oncogene (GRO), IGF-binding protein 6 (IGFBP-6), macrophage migration inhibitory factor, TIMP-2, VEGF-D, macrophage inflammatory protein 1 α , IL-8, tumor necrosis factor-related apoptosis-inducing ligand receptor 4, IL-12 p40, and macrophage-stimulating protein expressions were increased over time and were very high or high at 24 h, but not

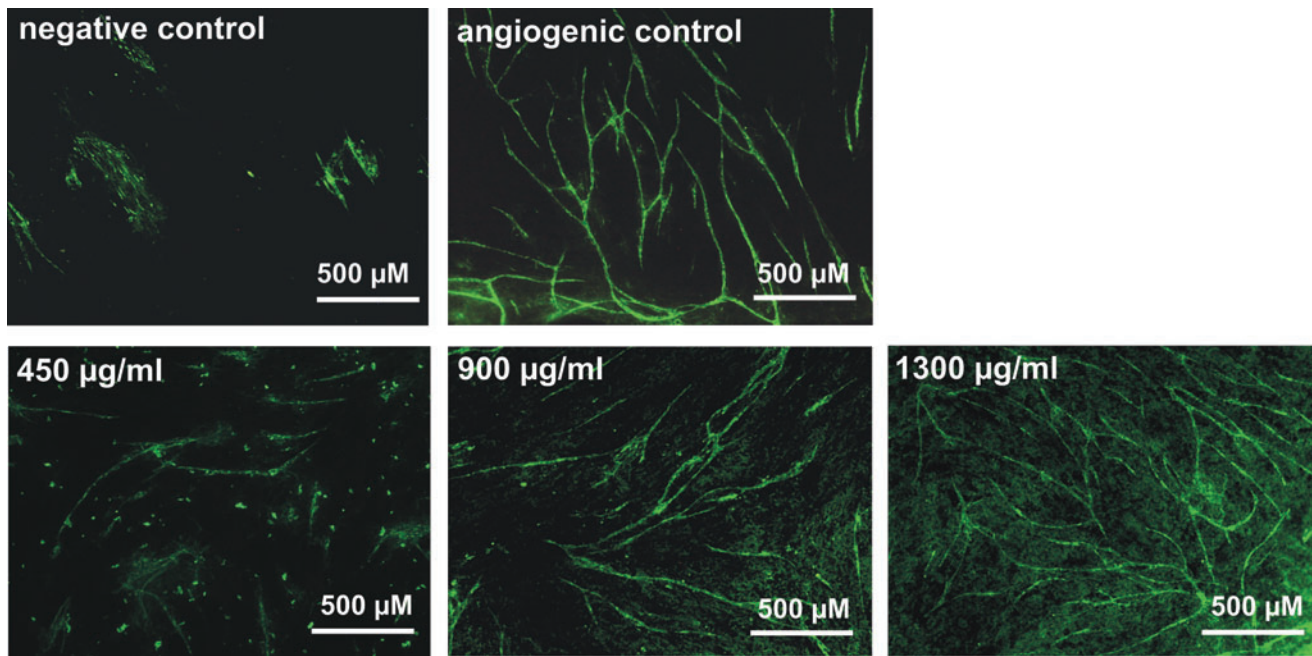


FIG. 3. Tubule formation of HUVECs in the presence of ATE in angiogenesis coculture assay. Different concentrations of ATE were applied as cell culture supplement for dose-dependent induction in tubule formation of HUVECs. Cells were cultured for 7 days and immunostained with von Willebrand factor primary antibody (1:500; Sigma) and secondary antibody (polyclonal antibody to rabbit IgG FITC; Acris Antibodies). As a negative control, HUVEC culture medium was used. Positive control was treated with 10 ng/mL of VEGF and 1 ng/mL of bFGF. Scale bar: 500 μ M in each image. HUVEC, human umbilical vein endothelial cell. Color images available online at www.liebertonline.com/tea

at 1 h. IL-6 had a significant increase ($p < 0.001$) as tested with one-way ANOVA after semiquantitative analysis of chemiluminescence signals with FluorChem software v. 3.1. Moderate or low levels of numerous other cytokines, especially at 24 h, were released, as seen in Supplementary Figure S1.

Discussion

An effective method for angiogenesis induction is urgently needed in the tissue-engineered constructs. Adipose tissue will not develop unless there is adequate blood vessel formation in the developing tissue, and moreover, to function efficiently as an endocrine organ, an extensive vascularization in adipose tissue is needed.⁴² Adipose tissue itself secretes considerable amounts of multiple angiogenic and adipogenic factors in appropriate combination to the new adipose tissue to develop. Therefore, the freshly isolated adipose tissue can be suspected to be the most potential natural source of bioactive factors for inducing angiogenesis and adipogenesis.^{1,25} In fact, adipose stem cell-conditioned medium, widely studied previously,^{2,10,19,20,22,25,43,44} has shown to promote endothelial cell survival and tubule formation as well as to stimulate wound healing. In this study, our aim was to find out whether extracted components from mature adipose tissue are effective and useful in inducing especially angiogenesis to overcome the challenge of vascularization and further adipogenesis in soft tissue engineering. In contrast to previous studies, we specifically aimed at producing a cell-free, protein-rich extract from viable human adipose tissue cells with an easy extraction method. This procedure could be carried out, for example, in operation room conditions during surgery for autologous transfer of

these factors. The viability of those hASCs that were first used for ATE extraction was further tested in our study by isolating the cells from these adipose tissue specimens and culturing them for several passages. The hASCs had normal morphology and proliferation capacity (data not shown).

ATE was shown to contribute to adipogenesis when at least 200 μ g/mL of ATE was used in cell culture. The adipogenic differentiation of hASCs was seen less than after 1 week *in vitro* with ATE, and a vast majority, if not all, of the cells were homogeneously differentiating toward mature adipocytes, already with fairly low concentrations of ATE. ATE was shown to induce accelerated and even fourfold higher triglyceride accumulation compared with previously used adipogenic control treatment.^{2,45} Donor-dependent adipogenic ability was seen to some extent, but not the time-dependent ability. Important adipogenic genes, *ACBP*, as well as *PPAR γ* , suppression of which has been previously shown to impair angiogenesis,⁴⁶ were significantly increased (approximately three or sixfold, respectively) with ATE-differentiated cells compared with control. ATE is therefore efficient for reproduction and modeling of natural adipogenesis *in vitro*. This type of appropriate and reproducible *in vitro* model for adipogenesis does not yet exist.⁴⁷

Angiogenesis induction, on its behalf, required often higher concentrations of ATE, at least 450 μ g/mL, than adipogenesis induction. However, ATE-induced angiogenesis was dependent on donor and time, 24 h being occasionally more favorable to angiogenesis than 1 h. All the different batches of ATE were able to induce adipogenesis, but not necessarily angiogenesis, especially at lower concentrations. On the other hand, in some cases at 24 h time point, angiogenesis was effectively induced at 450 μ g/mL, but the

angiogenic effect did not increase with higher concentrations of ATE. This effect was also seen in our previous angiogenesis study with VEGF and bFGF,⁴¹ when increase in the growth factor concentration increased the tubule formation only until certain threshold. Therefore, the lower and upper limits of angiogenic potential of ATE need to be further carefully studied. Overall, ATE induced morphologically similar tubule formation as well-known inducers of angiogenesis, VEGF, and bFGF. The present study therefore confirms the previous results that combination of several inductive factors, and in appropriate combination, is beneficial for inducing angiogenesis.^{1,10,11} To further evaluate the responsible factors for the angiogenic and adipogenic effect in ATE, we analyzed a number of growth factors and cytokines involved in tubule formation, in stabilization of blood vessels, as well as in triglyceride accumulation, as reviewed, for example, by Semenza,¹¹ Ziche *et al.*,⁴⁸ and Papetti and Herman.⁴⁹ We found that ATE contains a wide variety of these crucial factors at levels that have also previously shown to be bioactive.^{10,20} The analyses revealed that ATE contains high levels of angiogenesis-stimulating (e.g., VEGF, IGF-I, bFGF, angiogenin, leptin, HGF, IL-6, IL-8) and -inhibiting factors (e.g., TIMP-2, Ang-2, adiponectin, also known as Acrp30),^{31,48} the most abundant being VEGF, IL-6, angiogenin, and adiponectin, of which VEGF and IL-6 expression was significantly increased over time, indicating the induced angiogenic potential of 24 h extract. The 24 h rise in VEGF concentration may be due to increased expression of IL-6 shown to upregulate VEGF production in adipose tissue,⁴² but also possibly due to partial hypoxia during incubation, known to induce VEGF production.¹⁰ ATE also contains a wide number of other cytokines affecting angiogenesis and/or adipogenesis, of which many (e.g., macrophage colony-stimulating factor, GRO, intercellular adhesion molecule 1, FGF-9, and IGFBP -3 and -6) were increased over time.

The previous studies on adipose stem cell cytokine content^{2,10,19,20,22,25,44,50} show a corresponding yet slightly different cytokine profile. Similar patterns of highly expressed factors in hASCs to our study were reported by Verseijden *et al.*,² Rehman *et al.*,¹⁰ Kilroy *et al.*,¹⁹ Traktuev *et al.*,²⁰ and Rubina *et al.*²² In contrast to Traktuev *et al.*,²⁰ IFN- γ , TNF- α , IL-8, bNGF, MCP-1, MCP-2, and leptin were detected in our study. In contrast to Rehman *et al.*,¹⁰ we hardly detected any TGF- β or granulocyte macrophage-stem cell factor in ATE. The differences compared with previous stem cell studies can be largely explained by the source of ATE, which, on the contrary to previous studies, contains several different cells of adipose tissue. This finding is supported by the previous literature data, according to which several ILs and TNF- α , the pro-inflammatory cytokines contributing to angiogenesis, are mainly secreted by inflammatory cells, especially macrophages.^{30,51} Adiponectin, a major adipogenic hormone reported to have a dual role as an inhibitor⁵² and stimulator^{53,54} of angiogenesis, as well as leptin, one of the major adipogenic and angiogenic hormone, is secreted by mature adipocytes.^{14,30,31,42} TIMPs, the inhibitors of angiogenesis and contributors to adipogenesis, are released from connective tissue cells and macrophages.⁵¹ In a previous study by Uriel *et al.*,⁵⁵ a rodent homogenized ATE was found to contain several basement membrane components; nidogen, collagen type IV, fibronectin and laminin α 4. This allows us to suspect that human ATE also contains several of these

basement membrane components, although this needs to be further studied.

The angiogenic and adipogenic factors are known to act in a cascade in different phases of the refined process of adipose tissue formation as reviewed, for example, by Papetti and Herman.⁴⁹ The possibility of modification of factor content is an interesting advantage of ATE. With ATE we possibly could stimulate different phases of angiogenesis and adipogenesis by a simple adjustment of the extraction time of ATE to be favorable to the release of certain factors. Moreover, ATE could also possibly be further tailored by precipitating single or few growth factors from the extract with presently available methods for further use.

The results suggest that ATE is more effective for both angiogenesis and adipogenesis induction than the presently available and previously tested methods. ATE contains all the most important angiogenic and adipogenic factors of mature adipose tissue. ATE is completely cell-free and therefore suspected to be nonimmunogenic, which could also allow allogeneic use of ATE. ATE has potential to be used to induce revascularization of ischemic tissues and to be used in tissue products that fail because of inadequate vasculature in, for example, soft tissue engineering for replacement of adipose tissue transplants, which do not function properly.⁵⁶ Moreover, ATE is efficient for modeling natural adipogenesis *in vitro* to be used in preclinical phase of drug discovery for efficacy testing of novel drugs for, for example, obesity and diabetes.

Conclusions

We here presented a novel cell-free angiogenesis- and adipogenesis-inducing agent, ATE, produced from mature human adipose tissue. ATE contains a wide variety of promoters of angiogenesis and adipogenesis and efficiently induces angiogenesis and adipogenesis *in vitro*. ATE has strong potential to be used for angiogenesis or adipogenesis induction in numerous tissue engineering applications *in vitro* and *in vivo*.

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Disclosure Statement

No competing financial interests exist. Patent is pending (WO/2010/026299).

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Bioactive Acellular Implant Induces Angiogenesis and Adipogenesis and Sustained Soft Tissue Restoration *In Vivo*

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Soft tissue defects resulting from trauma, tumor resection, or congenital causes provide a challenging problem to reconstructive surgery and tissue engineering. Current therapeutic procedures lack the ability to induce rapid formation of neovascularization. Therefore, to date, no adequate application for the reconstruction of soft tissue defects is available. We have previously shown that bioactive factors extracted from adipose tissue (adipose tissue extract [ATE]) induce both adipogenesis and angiogenesis *in vitro*. These bioactive factors were incorporated into hyaluronan (HA) hydrogel, and the ATE-HA implant-induced angiogenesis and adipogenesis were studied. The developed implant was shown to gradually release the bioactive factors, and the presence of the implant in human adipose stem cell culture was able to induce adipogenic differentiation as evaluated by Oil-red-O staining. In animal experiments, the implants were placed under dorsal subcutis of rodents. Either rat-(rATE, allograft) or human- (hATE, xenograft) derived ATE was incorporated into implants. Local inflammation reactions, angiogenesis, and adipogenesis were followed from 1 week to 40 weeks. Angiogenesis was assessed by microvessel density analysis; adipogenesis was assessed by automated image analysis, and immunological effects by immunostaining and counting inflammatory cells. The key requirements for soft tissue replacement—host compatibility, bioactivity, and sustainability—were all achieved with the novel ATE-HA implant. This acellular implant induced microvessel induction early after implantation and adipose tissue deposition from 12 weeks onward as well as subcutaneous tissue volume increase. The ATE-HA implant was replaced by mature adipose tissue with capillaries, nerve bundles, and healthy connective tissue without local inflammation or capsule formation. The large fat pads remained in tissue until the end of the follow-up time, for 9 months. No adverse effects were detected at the site of implantation, and according to irritating ranking, the ATE-implant was considered to have excellent biocompatibility. The results demonstrate that an acellular HA hydrogel implant induces significant increase in adipogenesis and angiogenesis *in vivo* compared to the plain HA implant, and ATE has excellent potential for use in tissue engineering for sustained reconstruction of soft tissue defects.

Introduction

SOFT TISSUE ENGINEERING seeks to fabricate replacement parts for trauma- or disease-related soft tissue defects (burns, scars, and chronic wounds), for surgical resections, and for congenital malformations.^{1–3} In addition, cosmetic use, such as filling of facial wrinkles, is an important application of reconstituted soft tissue.^{1–3} Soft tissue defects and chronic wounds are a medical and economic challenge to the healthcare system,^{4,5} and there is an urgent clinical need for

an adipose tissue substitute that would induce rapid vascularization and sustained formation of adipose tissue.² Despite the progress in soft tissue engineering over the last years, tissue-engineered adipose tissue is still a great challenge, and adequate application for the reconstruction of soft tissue does not exist.^{6,7}

Current therapeutic procedures for soft tissue replacement include filling the defects with synthetic or natural biomaterials, using autologous fat grafts or stem cell therapy.^{2,8–10} Synthetic materials induce unwanted allergic reactions, and

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fat grafts have drawbacks such as resorption of transplants.¹⁰ Stem cell therapy is promising, although time consuming, and only autologous transfer of cells is possible.^{9,10} The adequate induction of angiogenesis is a prerequisite for successful adipose tissue reconstruction.^{11–13} It was recently reported that in tissue-engineered vascular grafts, the neovasculature was solely derived from the host cells via vascular remodeling, not from implanted cells.^{14,15} The cells of the tissue-engineered vascular graft actually promoted the host tissue regeneration instead of themselves differentiating into the vessels.¹⁶ The key inductive molecules would therefore be sufficient for adequate induction of vascular development.¹⁵ In fact, an acellular soft tissue substitute that would induce its own replacement would be advantageous to soft tissue engineering.^{14,17,18} Inductive adipogenesis would not require stem cell harvest from the patient, and an acellular alternative would prevent severe immune responses and could allow the alloplastic use of the implant.^{1,17,19–24} Several studies have already been performed to provide a suitable microenvironment for the formation of blood vessels and adipose tissue without the need to transplant cells. This has been tried by incorporating growth factors or extracellular matrix proteins into biomaterials.^{14,17,20,25–28} The controlled release of multiple angiogenic factors has shown to promote tissue regeneration at the site of the implantation.^{14,29,30} However, the creation of the optimal environment for inductive adipogenesis has been difficult, especially in long-term.⁸ Overall, all current therapy approaches for soft tissue induction, mostly due to inadequate vascularization,^{13,28,31} fail to produce long-term satisfactory and repeatable results,⁸ and therefore the sizes of the tissue-engineered implants are limited.^{31–33}

Adipose tissue is known to be a very active endocrine organ and an excellent source of growth factors and cytokines,^{30,34,35} and it can be thought to bear excellent potential for adipose tissue induction.³⁴ We have recently characterized and developed the process to extract growth factors and cytokines from adipose tissue (the adipose tissue extract [ATE]), and have shown that ATE is capable of inducing effectively both angiogenesis and adipogenesis *in vitro*, and has therefore potential to be used in soft tissue engineering.³⁴

The main objective of the current study was to create an acellular implant by incorporating ATE into a biodegradable biomaterial that would release these angiogenic and adipogenic substances locally in tissue. The ATE was combined with hyaluronan (HA) hydrogel and implanted subcutaneously in rodents. HA is a glycosaminoglycan and a natural component of the extracellular matrix that takes part in binding growth factors and cytokines,⁹ is known to modulate inflammation and tissue repair³⁶ and to promote angiogenesis,^{37,38} and is therefore considered a very potential material for soft tissue engineering.³⁶ HA has been previously used, for example, for inducing formation of bone and cartilage²⁶ and soft tissue.^{8,31} Several FDA-approved injectable HA hydrogel materials are already in clinical use, and the adverse reactions of HA derivatives have been minimal.^{39,40}

In the current study, the local effects after implantation of the grafts, inflammation reactions and induction of adipogenesis and angiogenesis, were followed. The efficacy and immunological effects were evaluated both short term and long term (from 1 to 40 weeks) *in vivo*, and both from allo-

graft (rat ATE-HA implant in rat) and xenograft (human ATE-HA implant in rat). The present study shows that acellular HA implant with ATE induces significantly more angiogenesis and adipose tissue formation in tissue than the control HA implant. The ATE implants are regarded as nonirritant, and the effects of implantation are sustained, at least for 9 months.

Materials and Methods

The adipose tissue samples were obtained from surgical operations with informed consents at the Tampere University Hospital, Tampere, Finland, in accordance with the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland (R03058). All animal experiments were performed according to the Finnish animal protection laws and approved by the Department for Social Welfare and Health Services of the State Provincial Office of Western Finland.

ATE preparation

Human adipose tissue specimens were obtained as subcutaneous tissue samples from surgical operations, and rat as subcutaneous fat adipose tissue specimens were obtained from sacrificed rats. Adipose tissue was cut into small pieces and transferred into 50-mL tubes (Sarstedt). Equal volume of the Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 (DMEM/F12; Gibco, Invitrogen), without supplements was added into the tube and left for 24 h for growth factor secretion at 37°C in a CO₂ incubator. The tube was gently shaken during incubation. The extracts were collected, centrifuged at 12,000 rpm for 5 min, and sterile-filtered through a 0.22-μm filter (Sarstedt). The aliquots were stored at –20°C before use.

Measurement of protein concentration

ATE samples, collected at 24 h, and phosphate-buffered saline (PBS) samples, from the protein release study, collected at several time points were analyzed for the total protein concentration with the BCA protein Assay Kit (Pierce Biotechnology).

Preparation of implants

The resulting sterile ATE, either from (1) human, hATE, or from (2) rat, rATE, was mixed with HA (nonanimal-derived partially cross-linked hyaluronic acid Restylane; Q-Med) in such a ratio that the implant contained 57% ATE and 43% HA hydrogel. As a control implant, HA with incorporated PBS was used, with corresponding volumes of HA (43%) and PBS (57%). The final human ATE concentration in the implant was 1.48 mg/mL of hydrogel (148 μg in each implant), and the final rat ATE concentration in the implant was 0.75 mg/mL of hydrogel (75 μg in each implant).

Isolation and culture of human adipose stem cells

Stem cells were isolated as described previously.^{34,41–43} Briefly, human adipose tissue specimens were cut into pieces and enzymatically digested with 0.05% collagenase I (Invitrogen). The digested tissue was centrifuged at 600 g for 10 min and filtered through 100- and 40-μm filters (Sarstedt). Human adipose stem cells (hASCs) were seeded and cultured

in DMEM/F12 supplemented with 1% L-glutamine (L-glut; Gibco), 1% antibiotic-antimycotic mixture (AB/AM; Gibco), and 15% human serum (Lonza).

Protein release studies

The protein release from the implants was tested by incubating the implants in PBS at 37°C up to 3 weeks. The 48-well plate inserts (Scaffdex Oy) were filled with HA-ATE hydrogels, prepared as described above, and put on the top of PBS-filled 48-well plate wells (Nunc). Aliquots of PBS were collected at predetermined time points and analyzed for total protein concentration with BCA protein Assay (Pierce Biotechnology).

Adipose stem cell differentiation with bioactive HA implant

To study the adipogenic effect of bioactive implants in cell culture, hASCs were plated in 48-well plates at early passages (p1–p3) at a density of 10,000 cells/cm². Cells were seeded and cultured in the hASC culture medium as described above. The next day, the hATE implants were created as described above. The 48-well inserts (Scaffdex) were filled with hATE-HA hydrogel and placed into cell culture wells. The medium was changed twice during the study. Cells were cultured for 6 days and examined for lipid accumulation with Oil-red-O (ORO; Sigma) staining.

ORO staining

Lipid accumulation was assessed at 6 days after the onset of the differentiation experiment by using ORO staining. ORO staining was performed as described previously.³⁴ Briefly, hASCs were fixed with 4% paraformaldehyde (Sigma) for 20 min, incubated with 60% isopropanol for 2 to 5 min, and stained with 3:2 diluted and filtered 0.5% ORO stock solution (Merck & Co., Inc.; prepared in 100% isopropanol) for 5 min. Images were taken with the Nikon Eclipse TS-100 microscope (Nikon) equipped with the Nikon DS Camera Control Unit DS L-1 (Nikon). The images were processed with Adobe Photoshop CS3 software (Adobe Systems) and Corel Draw software 10.0 (Corel Corporation).

Animal model of acellular soft tissue induction

Twenty-six male Sprague-Dawley rats (weight 325 ± 75 g, average age of 8 weeks) were housed per 2, with 12 h light and 12 h darkness, and fed pelleted diet and water *ad libitum*. Rats were anesthetized with a mixture of Domitor (medetomidin hydrochloride; Orion Pharma) 1 mg/mL (0.5 mg/kg) and Ketalar (ketamin hydrochloride; Pfizer) 10 mg/mL (75 mg/kg). Three different types of hyaluronic acid implants, each incorporated with different substances, were created as described above. About 100 µL of the resulting gel mixture was immediately injected with a 1-mL syringe and a 27-gauge needle (Terumo Europe) into dorsal subcutis of rat. Implants were carefully injected in between the upper subcutis and the muscle layer into rat dorsal subcutis. Three animals in each time point were used (*n* = 3); three constructs per rat were implanted, and the places of implants were varied in different time points. The injected implant was ~8 mm in diameter. Implants were left under subcutis for 1,

4, 12, 20, or 40 weeks, after which animals were sacrificed. The remaining implant and its surrounding tissue were carefully cut from the implant site in the dorsal subcutis and further processed for histological analysis.

Hematoxylin and eosin staining

The histological specimens were cut into 1-mm² pieces and fixed in 4% paraformaldehyde overnight, dehydrated with a graded ethanol series, and embedded in paraffin. Specimens were cut into 5-µm-thick slices with a microtome (Microm HM 430, Microm GmbH) and stained with hematoxylin and eosin (H&E) for histology. Briefly, samples were deparaffinized by treating them three times for 5 min each with xylene (VWR International, LLC) and after that with graded laskeva ethanol series. The samples were stained for 10 min with Mayer Hematoxylin or Papanicolauslösung 1a Harris' Hämatoxylinlösung (Merck KGaA), rinsed with tap water 10 min and with distilled water 2 min, then stained with 1% eosin for 15 s, and rinsed with tap water and further with distilled water. The samples were then dehydrated with 94% and 100% ethanol, treated 3 × 5 min with xylene, and mounted with Entellan (Merck KGaA) for storage. Images were taken with a Nikon Microphot FXA microscope (Nikon) connected to a Sony DXC-S500 camera control unit and Sony Twain Driver Software 1.0 (Sony Europe Limited) and further processed with Adobe Photoshop CS 3 software (Adobe Systems) and Corel Draw software 10.0 (Corel Corporation). The microscopic images from H&E-stained subcutaneous tissue slices were merged with Canon Photo Stitch 3.1 (Canon).

Immunohistochemistry

The histological specimens were cut into 1-mm² pieces and fixed in 4% paraformaldehyde overnight, dehydrated with a graded ethanol series, and embedded in paraffin. Specimens were cut into 3- or 5-µm-thick slices with a microtome (Leica DM2000; Leica Instruments GmbH or Microm HM 430; Microm GmbH, respectively). Samples were deparaffinized with xylene (VWR International, LLC) 3 × 5 min each, rehydrated with graded ethanol series, and washed with distilled water.

For anti-von Willebrand Factor and for anti-Neurofilament-68 staining (anti-vWF produced in rabbit; Sigma; 1:5000 and anti-NF-68 produced in mouse; Sigma; 1:500, respectively, both 4°C overnight), tissue samples were treated 15 min with 0.5% hydrogen peroxide (Mallinckrodt Baker, Inc.) in methanol (Mallinckrodt Baker, Inc.), microwave oven-treated 10 min in a preboiled 0.01 M sodium citrate buffer, pH 6.0, cooled 20 min in a sodium citrate buffer, and treated with the Histostain Plus Broad Spectrum Detection Kit (Zymed Laboratories, Inc., Invitrogen). The color was developed with a DAB Substrate kit (Zymed Laboratories) for 5 min. The samples were counterstained for 5 s with Mayer Hematoxylin and mounted with Entellan (Merck KGaA).

For anti-CD3 staining (CD3 [Clone SP7] rabbit monoclonal antibody; Thermo Fisher Scientific; 1:100, 90 min at room temperature), the samples were microwave oven-treated 2 × 7 min in 10 mM Tris-HCl, 1 mM EDTA, pH 9.0., cooled for 20 min in Tris-EDTA buffer, and then treated with the DAKO EnVision™ + System, HRP kit (DakoCytomation).

For anti-CD79a staining (mouse anti-human Cd79a; AbD Serotec, MorphoSys AG; 1:500, 4) and for anti-CD68 staining (monoclonal mouse anti-rat CD68; AbD Serotec, MorphoSys AG; 1:100, in 0.1% BSA in TBS, 4°C overnight), samples were treated with the Mouse on Rat HRP Polymer Kit (Biocare Medical). The color was developed with the DAKO Cyto-mation Liquid DAB Substrate Chromogen System (Dako), and samples were counterstained with Papanicolauslösung 1a Harris' Hämatoxylinlösung (Merck) and mounted with Pertex (HistoLab Products Ab).

The results were analyzed with a Leica DM 2000 microscope (Leica) from five different fields from three animals per each time point. Images were taken with a Nikon Microphot FXA microscope (Nikon) connected to a Sony DXC-S500 camera control unit and Sony Twain Driver Software 1.0 (Sony Europe Limited) and processed for publication with Adobe Photoshop software CS3 (Adobe Systems), Corel Draw software 10.0 (Corel Corporation), and Canon Photo Stitch 2.0. (Canon).

Determining the capillary density

The number of formed capillaries was calculated microscopically from three animals per time point (each containing three different types of implants), and from five different random high-power fields (hpf, 400×) in each implant at the implant-tissue interface with a Leica DM 2000 microscope (Leica Microsystems GmbH). The capillaries were confirmed to be stained positive for vWf, and the capillaries needed to have lumen that was visible in hpf (400× magnification). Both capillary proliferation and larger arteriole-like and venule-like vessels were counted.

Automated quantitative analysis of the adipose tissue formation

To quantitate the adipose tissue formation in rat subcutis, an automated computer-assisted analysis tool was earlier developed for fat deposit detection. The applicability of the analysis tool for quantification of fat and for evaluating the differences in the fat accumulation between time has been previously described.⁴⁴ Briefly, before analysis, the samples were stained as described above in H&E staining. H&E-stained slides were photographed with a Nikon Microphot FXA microscope (Nikon) connected to a Sony DXC-S500 camera control unit and Sony Twain Driver Software 1.0 (Sony Europe Limited) and analyzed with the analysis tool without any preprocessing to the images. The analysis tool estimates the area of fat coverage by segmenting the hue component of the image with dual thresholding. Size constraints were applied to remove small, fractioned areas and excessively large areas, which typically belong to background. Finally, the user was allowed to modify the segmentation result by removing any possible false detection and by adding missing areas. Adding of new areas was implemented using the seeded region growing where the user gives the seed point and the area grows around this point.⁴⁴

Measurement of the tissue expansion

The H&E-stained histological specimens were measured with a Nikon TS-100 microscope using Nikon TS Elements

software. The total vertical height of the rat dorsal subcutis sample (from the muscle layer to the epithelial layer) was measured from each implant from two different sites at a 40-week time point.

Evaluation of the local immunological effects of implantation

The local biological effects were quantitatively scored after immunohistochemical staining as described previously^{45,46} and according to the International Organization of Standardization (ISO) Standard ISO-10993-6:2007 "Tests for local effects of implantation," when applicable. The response was evaluated per hpf (400×) from five different fields and from three different animals per time point. The inflammation score criteria were (as modified from ISO 10993-6:2007) as follows: 0=no cell type detected; 1=rare; 1–5/hpf (except for giant cells, 1–2/hpf); 2=5–10/hpf (except for giant cells, 3–5/hpf); 3=heavy infiltrate; 4=packed. The final average irritating ranking (as described in ISO 10993-6:2007) was obtained from the average of overall scores.

- nonirritant (0.0–2.9)
- slight irritant (3.0–8.9)
- moderate irritant (9.0–15.0)
- severe irritant (>15)

The final active inflammatory changes were multiplied by two to get the final irritation ranking.

Statistical analysis

All statistical analyses were performed and graphs processed with GraphPad Prism 5 (GraphPad Software, Inc.). Samples were subjected to one-way analysis of variance followed by appropriate post-tests; for the capillary formation and adipose tissue formation, Tukey's and Dunnett's post-tests, and for tissue thicknesses, Tukey's post-test. The results were considered significant when * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Results

Protein release from the implants

The protein release of the designed implants was tested by incubating the implants with PBS as well as incubating the implants with ASCs. The ATE-containing implants were shown to release protein when incubated in PBS up to 3 weeks, whereas control implants did not show any protein release. The results are seen in Figure 1A. The protein released from the implants was able to induce adipogenesis in hASC culture as stained with ORO after 6 days of culture (Fig. 1B).

Histological evaluation of the local effects of implantation in tissue

The effects of implantation were evaluated with local tissue histology after H&E staining. The evaluated time points were 1, 4, 12, 20, and 40 weeks. The results are shown in Figure 2.

At 1 week, cell penetration into all of the implants was seen. Capillary proliferation with small newly formed capillaries was extensively present with both rATE-HA and

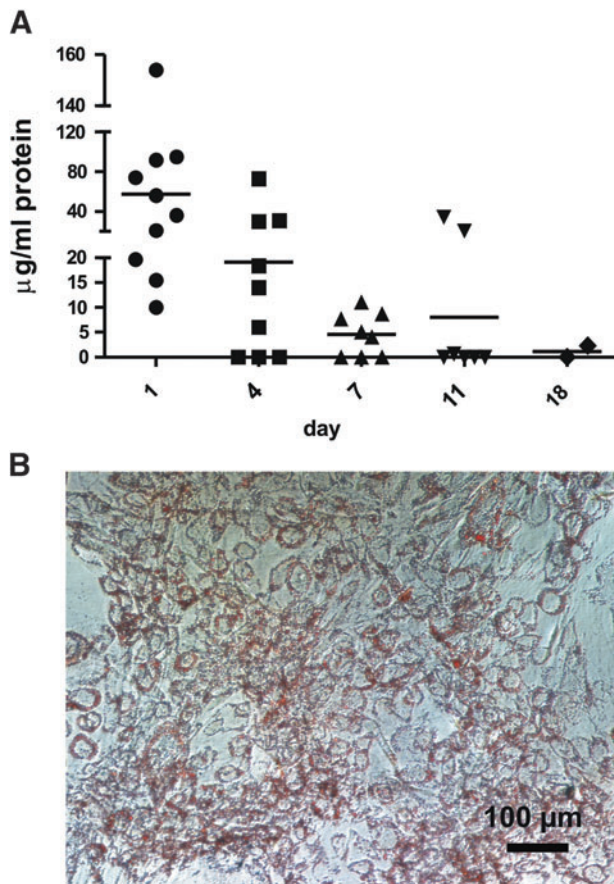


FIG. 1. The HA hydrogel protein release. **(A)** The protein release from the hATE-HA implant as measured with BCA protein assay kit. **(B)** The image of Oil-red-O-stained triglyceride accumulation in hASCs after being cultured with the HA implant for 6 days. Scale bar: 100 μm . ATE, adipose tissue extract; HA, hyaluronan; hASC, human adipose stem cell. Color images available online at www.liebertpub.com/tea

hATE-HA implants (Fig. 2B, C, respectively) at the implant-tissue interface. In rATE, also larger arteriole-like and venule-like vessels were seen next to the implant (Fig. 2B). The vessels were higher in number as well as in the diameter with rATE-HA and hATE-HA implants, compared to controls. Very small adipose tissue deposits, indicating triglyceride accumulation, were also seen (Fig. 2B). The control implant did not present any relevant tissue changes (Fig. 2A).

At 4 weeks, the tissue histology resembled that of 1 week. Vast capillary infiltration was seen with both rATE-HA and hATE-HA implants at the implant-tissue interface, close to the degrading hydrogel, and several arteriole-like and venule-like structures and a lot of small fat deposits were seen. Close to the implant, large vessels were also present. Only few larger capillaries were detected in the control implant (Fig. 2D).

At 12 weeks, few larger vessels and small fat deposits were seen in the control HA implant (Fig. 2G). In rATE-HA implant, dense, large, well-vascularized fat pads were already seen (Fig. 2H). Capillaries were evenly distributed throughout the fat tissue. The newly formed fat pads were often seen to be located apart from the native fat tissue, at the initial implant injection site. Inside the fat pads, vasculari-

zation and nerve bundles were visible. The hATE-HA implant (Fig. 2I) was histologically similar to the rATE-HA implant; however, the amount of fat was more extensive in the rATE-HA implant than in the hATE-HA implant.

At 20 weeks, the tissue histology was similar to 12 weeks, except that more adipose tissue had accumulated in the rATE-HA (Fig. 2K) and hATE-HA (Fig. 2L) implants. More adipose tissue was seen in the rATE (Fig. 2K) than in the hATE (Fig. 2L). The rATE-HA implants had induced large fat pad formations, seen as several layers of adipose tissue. All implants, the rATE-HA implant, hATE-HA implant, and control implant, were still present in tissue, and they were equally degraded.

At 40 weeks, the control implants were completely degraded. These implants had induced some adipogenesis, more than was seen in the control tissue. Two of the rATE-HA implants (out of three animals) were degraded completely. The degradation of the control implant was faster than the rATE-HA and hATE-HA implants. The hATE-HA implant degradation rate was decreased compared to other implants, as all of the implants still partly remained in tissue at 40 weeks. However, no capsule formation was seen, only a narrow layer of fibrosis occasionally. The large fat pad formations, seen also already at 20 weeks, and induced especially by rATE-HA implants, were present at 40 weeks, and the adipose tissue seen was well vascularized. The histology of the newly formed mature adipose tissue was similar to normal tissue in all implants at 40 weeks, when tissue histology was compared between the control implant (Fig. 3A), rATE-HA implant (Fig. 3B), and hATE-HA implant (Fig. 3C). The accumulated adipose tissue in the implants resembled that of the endogenous tissue with a densely packed structure and hexagonal-shaped cells, accompanied with blood vessels.

When tissue thicknesses were microscopically evaluated, the HA implant (Fig. 3D, rATE-HA implant) was shown to induce increase in thickness of subcutis at 20 weeks compared to control implant (Fig. 3E) and control tissue (Fig. 3F). The change in tissue thickness was evaluated by measuring the thickness of the subcutis from histological samples at 40 weeks. The results are shown in Figure 3G. As statistically evaluated, all implants induced a significant increase in tissue thickness at 40 weeks compared to control tissue (control implant $**p < 0.01$; hATE-implant $***p < 0.001$; rATE-implant $***p < 0.001$). At 40 weeks, the tissue thickness was significantly higher in the rATE implant and the hATE implant (both $***p < 0.001$), compared to the control implant.

The capillary proliferation was confirmed with anti-von Willebrand factor staining at 4 weeks (Fig. 4A), and tissue innervation, seen often from 12 weeks onward as nerve bundles, was confirmed with antineurofilament 68 staining (Fig. 4B). Nerve bundles were not detected at earlier time points, but often at 12 to 40 weeks.

Quantification of angiogenesis and adipogenesis in vivo

Capillary density was evaluated from five different microscopic hpf (400 \times magnification) at 1 and 4 weeks (e.g., before adipose tissue accumulation) by calculating the capillaries manually under microscope. The results are shown in Figure 5A. The statistical analysis was performed at both time points between different implants (significance

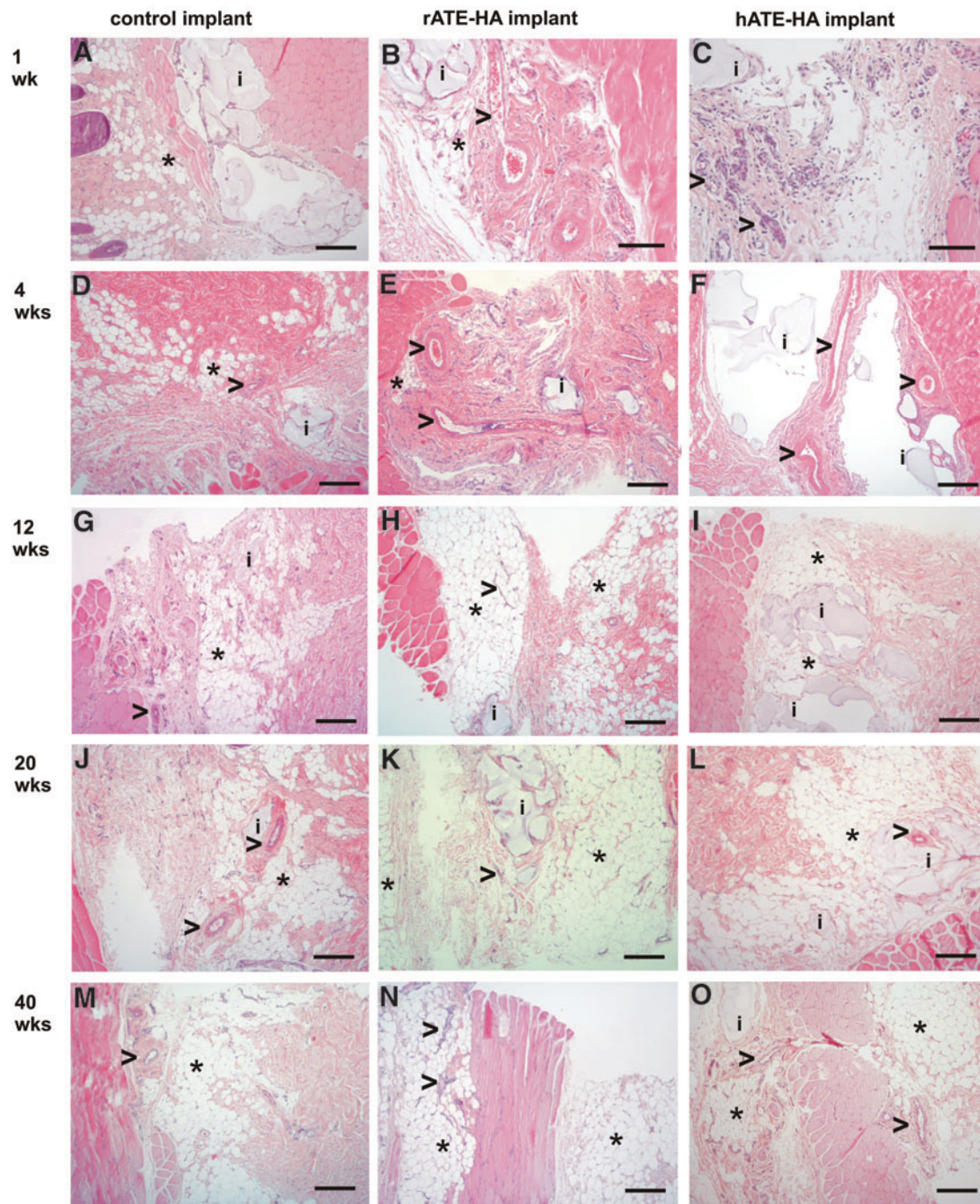


FIG. 2. The rat subcutaneous tissue histology at 1, 4, 12, 20, and 40 weeks after implantation of the rATE-HA implant, hATE-HA implant, or control (plain HA) implant. (A)–(C) implants at 1 week, (D)–(F) implants at 4 weeks, (G)–(I) implants at 12 weeks, (J)–(L) implants at 20 weeks and (M)–(O) implants at 40 weeks. The samples were H&E stained. Scale bar: 250 μ m, except for (B) and (C); scale bar: 100 μ m. i = implant, adipose tissue is shown with asterisks (*) and blood vessels with arrowheads (>). H&E, hematoxylin-eosin. Color images available online at www.liebertpub.com/tea

shown in Fig. 5A as asterisks) as well as by comparing each implant to their 1-week control (significance shown in Fig. 5A as hash signs). Both capillary proliferation and larger arteriole-like and venule-like vessels were counted. The overall number of vessels was highest at 1 week. The control implant blood vessel number was very similar at both time points. The hATE-HA implant induced significantly higher

capillary proliferation at 1 week ($p < 0.05$) and at 4 weeks ($p < 0.001$), compared to the respective control implants.

The adipose tissue was quantified with a specific analysis tool designed for the purpose and published previously.⁴⁴ The statistical analysis was performed at each time point between different implants (shown in Fig. 5B as asterisks) as well as by comparing each implant to their 1-week control (shown in

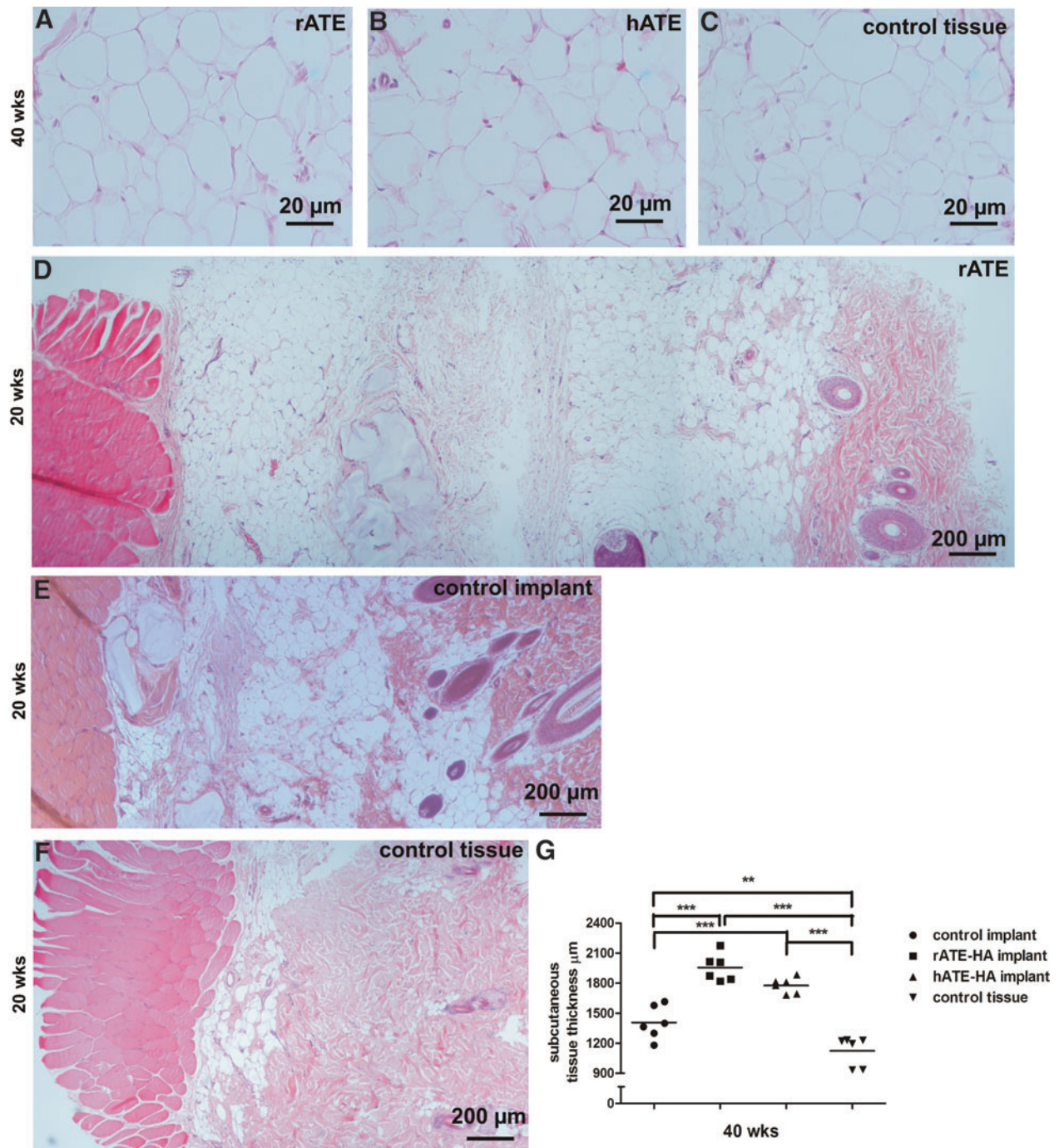


FIG. 3. The comparison of adipose tissue histology in H&E-stained images between rATE-HA implant (A), hATE-HA implant (B), and normal tissue (C) at 40 weeks. The comparison of the tissue thicknesses between rATE implant (D), control implant (E), and control tissue (F) at 20 weeks, as well as comparison of measured tissue thicknesses between implants at 40 weeks (G). The results in (G) represent individual observations and mean. Differences were considered significant when $**p < 0.01$ and $***p < 0.001$. Scale bar for (A), (B), and (C) 20 μm, and for (D), (E), and (F), scale bar: 200 μm. Color images available online at www.liebertpub.com/tea

Fig. 5B as hash signs). The number of adipose tissue (% from microscopic image, 40× magnification) was increased during the time; however, until 12 weeks, there was no significant increase in adipose tissue accumulation. At 12 weeks, the rATE-HA implant contained significantly more adipose tissue than the rATE-HA implant at 1 week ($p < 0.05$). At 20 weeks, all

(control implant, rATE-HA implant, and hATE-HA implant) had more adipose tissue than respective treatments at 1 week ($p < 0.05$, $p < 0.001$, and $p < 0.001$, respectively). Moreover, at 20 weeks, the rATE-HA implant and the hATE-HA implant had induced more adipose tissue accumulation than control tissue ($p < 0.05$ and $p < 0.01$, respectively). At 40 weeks, all implants, as

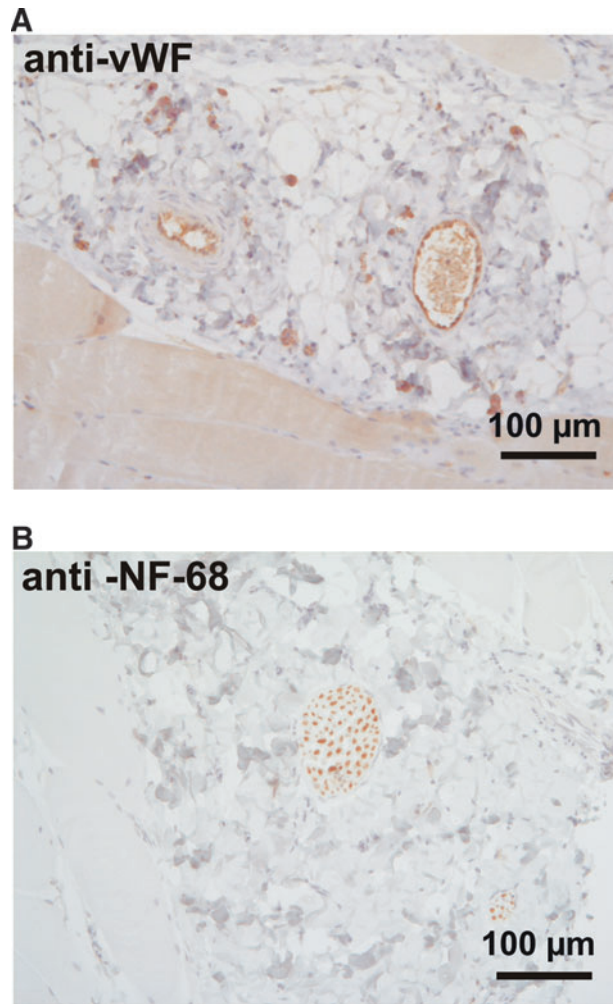


FIG. 4. (A) Anti-von Willebrand factor (anti-vWf) staining of capillaries) at 4 weeks and (B) anti-neurofilament -68 (anti-NF-68) staining of nerve bundles at 12 weeks in rat subcutaneous tissue after implantation of rATE-HA implant. Scale bar: 100 μm. Color images available online at www.liebertpub.com/tea

well as control tissue, contained more adipose tissue than respective treatments at 1 week (all highly significant, $p < 0.001$). However, at 40 weeks, the rATE-HA and hATE-HA implants had induced significantly more adipose tissue accumulation than the control implant ($p < 0.001$ with both implants) or control tissue ($p < 0.001$ and $p < 0.01$, respectively).

Immunological effects of implantation

The evaluation of immunological effects of implantation was modified from ISO-10993:6-2007. The results are scored and summarized in Table 1. To be able to perform irritating ranking as described in ISO 10993-6:2007, the active inflammatory changes were multiplied by two. Similar inflammatory scoring is also described previously.^{45,46} The irritation ranking was evaluated solely by immunological effects, not with induction of vascularization or fatty infiltrate, as these were the desired effects of implantation. No serious adverse tissue effects occurred. Some inflammatory

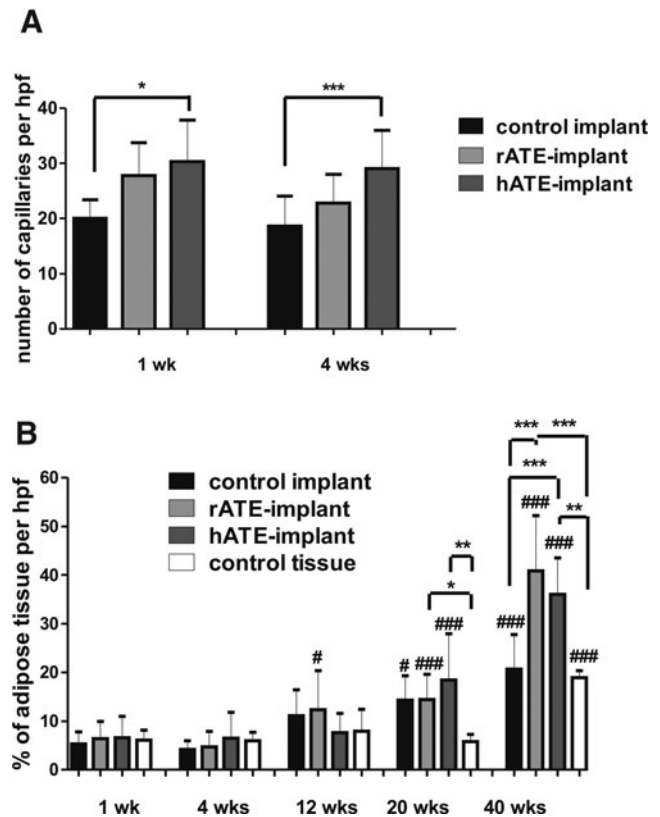


FIG. 5. The quantification of angiogenesis and adipogenesis after implantation of ATE-HA implants. (A) The capillary count in tissue at 1 week and at 4 weeks. The results were evaluated from five different high-powered fields (hpf, 400×) surrounding the implant and from three different animals in each time point. Differences were considered significant when $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. (B) The adipose tissue accumulation during time. The results were evaluated from 20 different images per each implant in each time point. The results are given as mean \pm SD. The implants were compared to each other in each time point (differences were considered significant when $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$). Each implant was also compared to their one week control (differences were considered significant when $#p < 0.05$, $##p < 0.01$, and $###p < 0.001$).

cell infiltration was seen in earlier time points. At week 1, the control implant showed moderate B-cell and mild macrophage infiltration, as well as the rATE-HA and hATE-HA implants showed mild infiltration of B-cells and macrophages. The polymorphonuclear cell infiltration was minimal throughout the study. During the time course, the control implant inflammatory effects were decreased and were completely disappeared at 40 weeks, when also the implant had degraded completely. The rATE-HA and hATE-HA implants were both present at 40 weeks, hATE-HA in each sample and rATE-HA occasionally, and they still showed some inflammatory cell infiltration at the time. No granuloma, fibrous capsule formation, giant cells, or necrosis were seen in any of the samples. Implants were seen to be often surrounded by a narrow band of fibroblasts, but after implant was degraded, no signs of fibrous bands were seen. Normal fibroblast proliferation was often

TABLE 1. HISTOLOGICAL SEMIQUANTITATIVE EVALUATION OF THE LOCAL EFFECTS OF IMPLANTATION

Inflammatory cell type	Implant response (average score) ^a											
	1 week			20 weeks			40 weeks					
	ctrl	HA	rATE+HA	hATE+HA	ctrl	HA	rATE+HA	hATE+HA	ctrl	HA	rATE+HA	hATE+HA
Polymorphonuclear cells (H&E)	0.4	0.3	0.3	0.3	0.4	0.3	0.2	0.1	0.2			
Lymphocytes (T-cells CD3)	1.2	2.2	1.9	1.1	0.5	0.7	0.7	0.5	0.6			
Plasma cells (B-cells and plasma B-cells CD79a)	3.6	2.7	2.6	2.5	2.1	1.7	1.1	1.6	2			
Macrophages (CD 68)	2.8	2.7	2.8	2.5	2.3	2.3	1.4	1.7	1.6			
Giant cells (H&E, CD68)	0	0	0	0	0	0	0	0	0			
Necrosis (H&E)	0	0	0	0	0	0	0	0	0			
Total	8	7.9	7.6	6.3	5.3	5.1	3.4	3.9	4.4			
Average total	1.3	1.3	1.3	1	0.9	0.8	0.6	0.7	0.7			
Total×2	16	15.8	15.2	12.5	10.5	10.1	6.8	7.9	8.8			
Average (test sample-control HA) ^b	—	0 ^c	0 ^c	—	0 ^c	0 ^c	—	1.1	2			

^aThe response has been evaluated per high-powered field (hpf, 400×) from five different fields surrounding the implant and from three different animals in each time point.

The inflammation score criteria were (as modified from ISO 10993-6:2007)

0=no cell type detected,

1=rare, 1–5/hpf (except for giant cells, 1–2/hpf),

2=5–10/hpf (except for giant cells, 3–5/hpf),

3=heavy infiltrate,

4=packed

^bThe average irritating ranking (as described in ISO 10993-6:2007) was as follows:

• nonirritant (0.0–2.9)

• slight irritant (3.0–8.9)

• moderate irritant (9.0–15.0)

• severe irritant (>15)

^cNegative difference was recorded as zero.

ATE, adipose tissue extract; HA, hyaluronan; ISO, International Organization of Standardization.

seen, and it was accompanied with capillary proliferation. According to the evaluation of local immunological effects, the implant was considered to be nonirritant compared to the control implant.

When statistically evaluated, none of the implants were found to be more irritating than others. The CD3- (T-cell) positive cell count was significantly higher in the rATE-HA implant at 1 week than with any other implant and in any other time point ($p<0.001$) as well as in the hATE-HA implant at 1 week compared to implants at 20 weeks ($p<0.05$) and 40 weeks ($p<0.01$). The CD68- (macrophage) positive cell infiltration was significantly higher in the control implant at 1 week and at 20 weeks compared to the control implant at 40 weeks ($p<0.01$ and $p<0.001$, respectively). Moreover, the rATE-HA and hATE-HA implants had significantly more macrophage infiltration at 1 week compared to the respective implants at week 40 ($p<0.05$ and $p<0.01$, respectively). The control implant had significantly more CD79a-positive cells at week 1 than at week 20. The polymorphonuclear cell count remained low in all implants throughout the study.

Discussion

Soft tissue engineering is a prominent research area, as soft tissue defects and chronic wounds are a medical and economic challenge to the healthcare system, and the demand is increasing every year.^{4,5} The ideal soft tissue replacement should induce rapid vascularization, induction of adipo-

genesis, and induce a permanent effect in tissue.² However, the current applications have shown only modest success, for example, some vascularization, but hardly any adipose tissue formation,¹⁴ and the optimal adipose tissue substitute remains to be discovered.

Our approach for the induction of capillary proliferation and adipose tissue accumulation was to create an *in vivo*-like environment for soft tissue formation as possible. In our previous work, we demonstrated that extracted factors from adipose tissue (ATE) induce angiogenesis and adipogenesis *in vitro*.³⁴ ATE is able to induce adipogenesis at $\geq 200 \mu\text{g/mL}$ protein and angiogenesis at $\geq 450 \mu\text{g/mL}$ protein.³⁴ ATE is a mixture of a wide variety of components of mature adipose tissue, of which the most abundant are, for example, vascular endothelial growth factor, fibroblast growth factor 2, adiponectin, angiogenin, interleukin 6, leptin, and insulin-like growth factor-binding protein 6.³⁴ These factors are known to have an inductive role on adipogenesis and/or angiogenesis (reviewed in Szasz and Webb).⁴⁷ In the present study, we demonstrate that both the induction of microvessels and adipose tissue can be effectively and permanently induced with these adipose tissue-derived factors *in vivo*. The key requirements for soft tissue replacement, as listed by Choi *et al*,² are host compatibility, bioactivity, and sustainability, all of which were achieved with the novel ATE-HA implant.

The ATE-HA implant induced extensive capillary proliferation early after implantation, and the induction was

significantly higher than the control implant. The induction of vascularization during the first 4 weeks was well in line with the protein release rate of the implants detected *in vitro*. The human ATE induced slightly more capillary formation than rat ATE in rat subcutaneous tissue. This may have been due to the higher concentration of hATE in the implant compared to rATE (rATE implant contained 75 µg protein and hATE implant 148 µg protein). However, as the rat ATE was overall slightly more efficient in adipose tissue accumulation in rat tissue than human ATE, one explanation may be that human factors act differently in rat adipose tissue remodeling than allogenic (rat) factors. In fact, it is known that there are differences between rodent and human adipose tissue.⁴⁸ In adipose tissue, key adipokines have differences in function in humans and rodents, as reviewed in Wang *et al.*⁴⁹ From 12 weeks upward, when also adipose tissue started to accumulate, the histology of the newly formed tissue vasculature resembled that of endogenous adipose tissue. The growth factor-induced neovascularization was extensive early after implantation compared to endogenous tissue, but during adipogenesis, the vessels were remodeled. During angiogenesis stimulus in tissue, the endothelial cells start to proliferate toward the stimulus, and a number of immature capillaries are formed. During vessel remodeling, the vessels that cannot become perfused are regressed,⁵⁰ resulting in normal tissue restoration. Similarly, the ATE-implant induced formation of adipose tissue with vasculature that was histologically comparable to the endogenous fat tissue. It is an important finding that ATE is able to induce efficient tissue restoration with normal vascular remodeling.

The adipose tissue deposition was abundant and microscopically observed as dense fat pads from 12 weeks onward with ATE-HA implants (significant increase compared to control tissue), and also, the subcutaneous tissue volume was increased. However, due to variation between animals, the adipose tissue increase was not statistically significant compared to the control implant, until at 40 weeks. The timing of adipose tissue appearance in ATE implants most likely reflects the release properties of the biomaterial used in the study. The inductive factors are first released by diffusion from the implant; however, later on, the factors are released simultaneously with the degradation of the material. The inductive effect could be possibly reached sooner, if a higher concentration of ATE was used, or moreover, different type of hyaluronic acid or other biomaterial was used. However, this remains to be further tested.

The rat ATE was overall slightly more efficient in adipose tissue induction in rat tissue than human ATE. However, both the rATE-HA implant and the hATE-HA implant were replaced by newly formed adipose tissue that had histological features of normal adipose tissue; capillaries, nerve bundles and normal connective tissue, and the fat pads remained in tissue until the follow-up time (9 months). Already at a 20-week time point, the 100 µL rat ATE implant had induced even a sevenfold increase in fat accumulation compared to control tissue (~0.4-mm fat layer in control tissue vs. even 2.8-mm fat layer in the rat ATE implant), and almost 2.5-fold induction compared to the control implant (~1.2-mm fat layer in the implant). Both ATE implants induced significantly thicker tissue than the control implant at 40 weeks. The sustained effect in this extent has not been

demonstrated previously by any of the applications tested, neither with hASC-seeded scaffolds nor with acellular scaffolds. For example, in a previous study by Hemmrich *et al.*,³⁷ blood vessels were formed, but hardly any mature fat was seen by 12 weeks postimplantation. In a 24-week study by Tsuji *et al.*,⁵¹ only modest *de novo* adipogenesis was seen. Overall, previous studies show some adipose tissue formation, but no vascularization,⁵² or limited longevity of the adipose tissue²⁰ and not enough information on long-lasting (over 3-month) effects.^{52–54}

Tissue fillers like hyaluronic acid are known to contribute to angiogenesis and adipogenesis,^{36–38} and this was also seen in our study, as the control implant did increase both capillary and adipose tissue formation in some extent. However, at 40 weeks, when the control implant had degraded, the control implant had not induced any more adipose tissue than what was seen in control tissue. There was an increase in the tissue thickness in the control implant, which was however often contributed by the connective tissue formation induced by the control implant. Therefore, the control implant did not induce mature adipose tissue formation, although mild adipose tissue induction compared to normal tissue. The control tissue also represented a significant change in adipose tissue accumulation between week 1 and 20 and/or 40 weeks. It is a well-known phenomenon and a normal feature presented in laboratory animals that laboratory animals gain weight during time when they get older.

The key feature of a soft tissue substitute, the good biocompatibility,² was achieved with the ATE-HA implant. No adverse reactions or fibrous capsule formation occurred, and when the local inflammatory cell response of the implants was observed, ATE-HA implants did not show increase in irritating effects over the control HA implant. At 1 week postimplantation, the T-cell marker CD3 was increased in the rATE-HA and hATE-HA implants, and on the other hand, the B-cell marker CD79a in the control implant and macrophage infiltration in all implants. This is most probably due to the tissue damage caused by the injection of the implant into the subcutaneous space. We cannot exclude the possibility that hyaluronic acid itself induces inflammatory cell infiltration; however, ATE does not have any additional effect on the number of inflammatory cells, and therefore can be considered to be biocompatible and safe. In fact, macrophage infiltration is reported to be intrinsic for adipose tissue formation.^{55–57} Macrophages are known promote angiogenesis,^{36,58} hASC differentiation into endothelial cells,⁴⁵ and even to transdifferentiate into adipocytes.⁵⁵ It is also noteworthy that there was no difference with the immunological reaction between allogenic (rat ATE in rat) and xenogenic (human ATE in rat) implants, indicating that the ATE is well tolerated.

The angiogenesis-/adipogenesis-inducing effect in rats was demonstrated with quite low doses of ATE. Each rATE implant contained 75 µg protein and each hATE implant 148 µg protein. In the current study, the injectable implant was administered only once, at the onset of the study. Repeated administration and increased dose of ATE could even enhance the inductive effect. As the potential cytokines are injected locally, the overall doses of adipogenic and angiogenic factors required are reduced. However, the optimal dose and efficacy, and administration schedule of ATE, are currently under further investigation, as the right combination of factors is

known to be crucial,^{28,30} rather than the maximal dose. The effects of simple ATE substance, if used without incorporation into scaffold, are currently under study, as well as the systemic toxicity of high concentrations of ATE. It also remains to be resolved whether the range of indications of ATE could be broadened, that is, whether the inductive effect of ATE applies to other tissues than soft tissue.

We have developed a new acellular approach to soft tissue engineering. ATE combined with HA hydrogel is able to induce vascularization and soft tissue formation in tissue. The key requirements for soft tissue replacement—host compatibility, bioactivity, and sustainability—are all achieved with the novel ATE-HA implant. The blood vessel induction in tissue is rapid, and the newly formed adipose tissue remains at least for 40 weeks and is histologically comparable to the native mature adipose tissue. The implant presents no immunological risks even when used in a xenogenic manner, and therefore is an important tool in creating an allogenic soft tissue substitute for patients. This bioactive implant is a potential replacement for the current soft tissue products to overcome the challenge of vascularization and to restore soft tissue permanently.

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Disclosure Statement

No competing financial interests exist. Patent is pending (WO/2010/026299).

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Adipose Stromal Cell Tubule Network Model Provides a Versatile Tool for Vascular Research and Tissue Engineering

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Key Words

Angiogenesis • Endothelial cells • In vitro differentiation • Mesenchymal stem cells

Abstract

The current limitation in designing three-dimensional tissue models is the lack of adequate vascularization with mature and stable vessels. Adipose tissue is known to secrete several angiogenic factors, and human adipose stromal cells (hASC) are known to promote vessel growth, maturation and stabilization. In this study, hASC were induced to angiogenesis with growth factor-enriched medium either in monoculture or in coculture with human umbilical vein endothelial cells (HUVEC) and analyzed for vascular, pericytic and smooth muscle cell markers. hASC and HUVEC cocultures showed an accelerated proliferation rate and the cells self-assembled, independent of the cell passage number, into multilayered three-dimensional tubular networks. The networks of hASC and HUVEC expressed endothelial markers, a complete basement membrane and vessel-supporting cells with contractile properties. A hASC and green fluorescence protein-HUVEC-infection model revealed that cocultures consisted of a mosaic of von Willebrand factor-positive cells derived from

both cell populations – hASC and HUVEC. hASC monoculture had passage- and donor-dependent ability to form tubular networks, with half of the cultures presenting tubule structures and basement membrane formation. Pericytic and smooth muscle cell markers were expressed in hASC monoculture even when tubules were absent. By combining the potential properties of hASC and features from the present angiogenesis assays, we generated a natural-like, xeno-free, prevascular-like network in vitro model with excellent reproducibility and minimal limitations in technical performance. This tubular network model is an excellent tool for studying cell interactions during vascular development, for chemical and drug testing and for developing natural-like, multilayered, vascularized, scaffold-free tissue models.

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Introduction

The current major limitation in designing three-dimensional vascularized tissue models and implantable tissue constructs is the lack of adequate vascularization in the construct and especially the maturation and stabilization of the vasculature [Moon and West, 2008; Rivron et

Abbreviations used in this paper

bFGF	basic fibroblast growth factor
COLIV	collagen IV
EGM-2	endothelial cell growth medium-2
GFP	green fluorescence protein
hASC	human adipose stromal cells
HS	human serum
HUVEC	human umbilical vein endothelial cells
IGF-I	insulin-like growth factor-I
PDGFR β	platelet-derived growth factor receptor- β
PECAM-1	platelet/endothelial cell adhesion molecule-1
RPLP0	ribosomal protein large P0
α SMA	α -smooth muscle actin
SMMHC	smooth muscle myosin heavy chain
VEGF	vascular endothelial growth factor
vWf	von Willebrand factor

al., 2008]. The vascular network with stable and functional vessels [Abramsson et al., 2002] is essentially needed to meet the nutritional and functional demands of the organ [Gerhardt and Betsholtz, 2003]. The vessel engineering should optimally produce natural-like and xeno-free vascular constructs. Preferably, no animal components or unnatural scaffold materials should be allowed in the tissue culture, the cultures should contain only growth factors and proteins that occur in tissue naturally, and vessels forming in culture should have features of maturing vessels, i.e. in addition to tubule structures, the pericyte recruitment, basement membrane formation and vessel-supporting layer of smooth muscle cells should appear.

Adipose tissue, an active endocrine organ, is known to secrete several angiogenic and adipogenic cytokines and growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and insulin-like growth factor-I (IGF-I) [Gimeno and Klamann, 2005; Rehman et al., 2004; Rubina et al., 2009; Sadat et al., 2007; Verseijden et al., 2009]. Moreover, human adipose stromal cells (hASC), isolated as a stromal-vascular fraction from the adipose tissue specimens, are known to have developmental plasticity both in vitro and in vivo [Planat-Benard et al., 2004; Rehman et al., 2004] and have the capacity to differentiate into multiple cell phenotypes such as adipose [Gimble and Guilak, 2003b; Zuk et al., 2001], smooth muscle [Traktuev et al., 2008] and endothelial cells [Miranville et al., 2004; Oswald et al., 2004; Pittenger et al., 1999; Planat-Benard et al., 2004; Wosnitza et al., 2007; Wu et al., 2007]. hASC are known to promote vessel growth, maturation and stabilization in vivo

[Amos et al., 2008; Cai et al., 2009; Covas et al., 2008; Traktuev et al., 2008; Zannettino et al., 2007] by secreting angiogenic factors and by differentiating into vessel lining supporting cells with pericytic properties [Amos et al., 2008; Kilroy et al., 2007; Traktuev et al., 2008]. The high proliferation and differentiation capacity of hASC makes them an ideal component for angiogenesis modeling, and the use of abundant adipose tissue as a source of endothelial cells would also be attractive for vessel engineering [Merfeld-Clauss et al., 2010; Miranville et al., 2004; Planat-Benard et al., 2004; Wu et al., 2007]. Moreover, understanding the role of angiogenesis in adipose tissue is important, as vasculature regulates both the adipose tissue mass development and adipose tissue reduction, and is also suggested to have an impact on regulating the mass of other adult tissues [Rupnick et al., 2002].

The angiogenic potential of hASC has widely been studied [Cao et al., 2005; Planat-Benard et al., 2004; Traktuev et al., 2008; Verseijden et al., 2009], alone or in co-culture with endothelial cells, in the presence of a supporting scaffold, e.g. collagen I, Matrigel or fibrin [Dietrich and Lelkes, 2006; Lai et al., 2009; Miranville et al., 2004; Oswald et al., 2004; Pittenger et al., 1999; Planat-Benard et al., 2004; Rubina et al., 2009; Verseijden et al., 2010; Wosnitza et al., 2007]. However, in general, in the previous methods, the capillary tubule formation has been quite modest or the test has contained animal-derived components. Moreover, several previous methods have focused only on the role of endothelial cell migration and proliferation during angiogenesis, i.e. tubule formation. However, as pericytes have a crucial role in the regulation of the microvessel growth, maturation and stabilization during angiogenesis [Amos et al., 2008; Benjamin et al., 1998], the endothelial tubule formation alone is not a sufficient measure of vascular growth and differentiation. The recruitment of pericytes along vascular tubules is an essential step in vessel maturation, needed to induce basement membrane formation and to prevent vessel regression [Gerhardt and Betsholtz, 2003]. The failure of the endothelial cells to establish mature structures in vitro is often due to an absence of stabilizing mural cells [Merfeld-Clauss et al., 2010]. Coculture models that have been used in several studies [Bishop et al., 1999; Donovan et al., 2001; Friis et al., 2003, 2005; Merfeld-Clauss et al., 2010; Montesano et al., 1993; Nicosia and Ottinetti, 1990] provide a useful method for studying angiogenesis in controlled conditions in vitro, with the advantage of being able to replicate some tissue-derived signaling [Rubina et al., 2009]. However, only a few studies [Merfeld-Clauss et al., 2010; Traktuev et al., 2008] have

characterized hASC and human umbilical vein endothelial cells (HUVEC) scaffold-free cocultures and their angiogenic properties or hASC monoculture angiogenic properties [Miranville et al., 2004].

Our specific aim was to develop a completely human-based, simplified and improved angiogenesis model, a prevascular-like network with properties of maturing vessels, that could be used for studying angiogenesis in vitro, and that would aid in the development of tissue models by improving target cell proliferation, survival and differentiation. In this prevascular-like network model, cells are induced with natural growth factors and allowed to self-assemble into tubular network and vascular supporting structures. We studied the hASC+HUVEC coculture model by combining some properties and analyses from previous studies [Friis et al., 2003; Merfeld-Clauss et al., 2010] and the fibroblast-HUVEC coculture angiogenesis assay previously validated in our laboratory [Sarkanen et al., 2011]. We induced the hASC+HUVEC coculture or hASC monoculture with naturally occurring angiogenic growth factors; epidermal growth factor, VEGF, bFGF and IGF-I [Mehta and Besner, 2007]. We evaluated the cultures for expression of vascular, pericytic and smooth muscle cell markers by immunocytochemistry and quantitative RT-PCR. We also studied the endothelial differentiation capacity of hASC in both hASC monoculture and a hASC and green fluorescence protein (GFP)-HUVEC-infection model. We produced an improved assay that, due to growth factor addition, induces a multilayered prevascular-like network with properties of maturing vessels that do not regress over time. We also showed that hASC, at low passages, are able to differentiate into endothelial-like cells alone and in hASC+HUVEC coculture. This improved and easy tubular network model is an excellent tool for studying cell interactions during vascular development, in drug and chemical screening, and most significantly, in designing vascularized tissue models in vitro or, as it is xeno-free, for creating vascularized tissue constructs for implantation.

Materials and Methods

The study was conducted in accordance with the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland (R03058, R08028). The adipose tissue samples were obtained from surgical operations and the human umbilical cords from scheduled cesarean sections with informed consents at the Tampere University Hospital, Tampere, Finland. The experiments complied with the current laws of Finland.

Isolation and Culture of hASCs

Stem cell isolation procedure was performed as described previously [Gimble and Guilak, 2003a; Hong et al., 2005; Niemela et al., 2007]. Briefly, human adipose tissue specimens were cut into small pieces, enzymatically digested with 0.05% collagenase I (Invitrogen, Paisley, UK) in Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 (Gibco, Invitrogen, Carlsbad, Calif., USA) for 60 min at 37°C in a gyratory water bath. The digested tissue was centrifuged at 600 g for 10 min at room temperature. The digested tissue was filtered through a 100- μ m filter (Sarstedt, Nümbrecht, Germany), centrifuged and filtered again through a 40- μ m filter (Sarstedt). Cells were seeded into 75-cm² flasks (Nunc EasyFlask™, Nunc, Roskilde, Denmark) in Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 supplemented with 1% L-glutamine (Gibco), 1% antibiotic-antimycotic mixture (Gibco) and 15% human serum (HS; Cambrex, East Rutherford, N.J., USA). The next day, cells were washed several times with PBS. The cells were maintained at 37°C under a 5% CO₂ air atmosphere at a constant humidity and medium was changed every 2–3 days. After having grown to confluency, cells were divided in a ratio of 1:2 to 1:3, or further used for cell culture studies.

Isolation and Culture of HUVEC

The HUVEC were isolated from human umbilical cord veins as described previously [Jaffe et al., 1973; Sarkanen et al., 2011]. Briefly, the cord was separated from the placenta and the umbilical vein was cannulated with a 20G needle and the needle was secured by clamping the cord over the needle with a surgical clamp. The vein was perfused with PBS to wash out blood and then the opposing end of the umbilical vein was clamped with a surgical clamp. Subsequently, the vein was infused with 0.05% collagenase I. The umbilical cord was incubated in a water bath at 37°C for 15 min. After incubation, the collagenase I solution containing HUVEC was flushed from the cord by perfusion with PBS into a 50 ml polypropylene tube (Sarstedt). The cells were centrifuged at 200 g for 10 min, washed once with medium, centrifuged again and resuspended in endothelial growth cell medium-2 (EGM-2), BulletKit medium (Lonza Group Ltd., Basel, Switzerland) and seeded into 75 cm² flasks. Medium was changed every 2–3 days and cells were divided when confluent. For assay controls, HUVEC were plated at 4,000 cells/cm² and cultured in EGM-2 BulletKit medium.

Flow Cytometric Surface Marker Expression Analysis of HUVEC

The HUVEC were harvested at passage 3 and analyzed with a fluorescence-activated cell sorter (FACSARIA, BD Biosciences, Erembodegem, Belgium). Monoclonal antibodies against CD13-PE (BD Biosciences), CD31-FITC, CD34-APC and CD44-FITC (Immunotools GmbH Friesoythe, Germany), CD117-APC and CD133-PE (Miltenyi Biotech, Bergisch Gladbach, Germany), CD144-PE, CD202b and VEGFR2 (R&D Systems Inc., Minn., USA) and CD63-PE (Abcam, Cambridge, UK) were used. Monoclonal antibodies against von Willebrand factor (vWF, Abcam) were conjugated with IgG-PE (CalTag Laboratories, Calif., USA). Ten thousand cells were analyzed per sample and the positive expression was defined as the level of fluorescence greater than 99% of the corresponding unstained cell sample.

Table 1. Oligonucleotide sequences used for RT-PCR experiments

Gene	Primer
Human <i>RPLP0</i>	Forward 5'-AATCTCCAGGGGCACCATT-3' Reverse 5'-CGCTGGCTCCCACTTTGT-3'
Human <i>PECAM-1</i> (CD31)	Forward 5'-TCATTTCTGGGATCCATATGCA-3' Reverse 5'-TGGGTGTAGAGAAGGATTCCGT-3'
Human <i>Angiopoietin 1</i>	Forward 5'-AGCTACCACCAACAACAGTG-3' Reverse 5'-CAAAGATTGACAAGGTTGTGG-3'
Human <i>Caldesmon</i>	Forward 5'-AAGAATCCTTGGGACAGGTGAC-3' Reverse 5'-GTGGTGGTTGTCTTGGCCTC-3'

Lentivirus Infection

Lentiviral construct pLKO-MISSION-Bright-GFP was purchased from Biomedicum Genomics (BMGen, Biomedicum Helsinki, Helsinki, Finland). The infection was carried out with HUVEC at low passages with 300 µl of pLKO-MISSION-Bright-GFP in 1 ml EGM-2 BulletKit medium (1 U/ml). Virus infection was accelerated with 8 µg/ml hexadimethrine bromide (Sigma). After 24 h of incubation, medium was replaced with fresh EGM-2 medium. Highly fluorescent clones were selected with cloning rings and further selected with dilution cloning to obtain pure GFP-HUVEC culture. After expanding the infected HUVEC, they were used for hASC and HUVEC coculture assay as described below.

hASC Monoculture Assay

hASC (six different cell lines derived from different donors, passages 1–7 used) were seeded in EGM-2 BulletKit medium into 48-well plates (Nunc™ Multidishes, Nunc, Roskilde, Denmark) at a density of 20,000 cells/cm². Cells were induced for either 3 or 6 days in EGM-2 BulletKit medium, a commercially available growth factor-enriched medium containing epidermal growth factor, VEGF, bFGF, IGF-I, ascorbic acid, heparin, 0.1% gentamicin/amphotericin-B and 2% FBS, or in DMEM/F-12 medium supplemented with 15% HS, 1 mM L-glutamine and 1% antibiotic-antimycotic mixture. Medium was changed and the treatments applied once to cells cultured for 3 days and twice to cells cultured for 6 days.

hASC and HUVEC Coculture Assay

hASC (21 different cell lines derived from different donors, passages 1–7 used) were seeded in EGM-2 BulletKit (Lonza) culture medium into 48-well plates (Nunc™ Multidishes, Nunc, Roskilde, Denmark) or for confocal imaging into 8-well slides (Ibidi, Ibidi GmbH, Martinsried, Germany) at a density of 20,000 cells/cm². HUVEC, cultured as above (24 different cell lines derived from different donors, passages 1–6 used), were immediately carefully seeded on top of hASC at a density of 4,000 cells/cm². The seeding densities of hASC and HUVEC were chosen according to previous studies by us and others [Donovan et al., 2001; Friis et al., 2003; Sarkanen et al., 2011]. The day after plating, the differentiation treatments were applied to hASC+HUVEC coculture. Treatments were (all in duplicate wells): (1) EGM-2 BulletKit medium (composition as described

above in hASC monoculture assay); (2) EGM-2 BulletKit – medium in which 2% FBS was replaced with 2% HS; (3) EGM-2 BulletKit – medium without serum, and (4) endothelial cell basal medium-2 (Lonza) containing no growth factors, supplemented with 0.1% gentamicin, 2% FBS and 1 mM L-glutamine. Cells were cultured for either 3 or 6 days prior to immunocytochemistry or quantitative RT-PCR. Medium was changed and the treatments applied once to cells cultured for 3 days and twice to cells cultured for 6 days.

Quantitative RT-PCR

The primers used for angiogenesis and blood vessel maturation genes – platelet/endothelial cell adhesion molecule-1 (*PECAM-1*, also referred to as CD31); the blood vessel stabilizing signal molecule angiopoietin 1 (*Angiopoietin 1*); the smooth muscle cell microfilament protein regulating cell contraction, caldesmon (*Caldesmon*); and reference gene ribosomal protein large P0 (*RPLP0*; all oligonucleotides from Oligomer Oy, Helsinki, Finland) – are shown in table 1. The total RNA from hASC or hASC+HUVEC was extracted from 3 or 6 days differentiated confluent cultures using TRIzol® (Invitrogen, Carlsbad, Calif., USA) following the manufacturer's protocol. cDNA was synthesized using High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, Calif., USA) according to manufacturer's instructions. Quantitative RT-PCR analysis was performed in a 96-well optical reaction plate using an ABI Prism 7000 sequence detector (Applied Biosystems). As the expression of *RPLP0* gene was stable between experimental conditions, we used this gene as the reference for data normalization. hASC grown in hASC culture medium were used as controls for gene expression for *Angiopoietin 1* and *Caldesmon* and HUVEC grown in EGM-2 for *PECAM-1*. Reactions were performed using SYBR Green PCR Master Mix kit (Applied Biosystems), 50 ng cDNA sample and 10 µM primers. The PCR conditions were as follows: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The target and reference genes were amplified in separate wells. All reactions were performed in duplicate. Relative RNA expression was calculated in comparison to *RPLP0* RNA expression for cell culture experiments using the Pfaffl method [Pfaffl, 2001]:

$$\text{ratio of relative expression} = \frac{(E_{\text{target}})^{\Delta\text{CP target (control-sample)}}}{(E_{\text{ref}})^{\Delta\text{CP ref (control-sample)}}}$$

Immunocytochemistry

The tubule formation was visualized with endothelial cell-specific antibody to vWf (anti-vWf primary antibody produced in rabbit, 1:500, Sigma). To evaluate human adipose stromal cell differentiation, parallel double immunofluorescence staining with α -vWf was performed. Primary antibody against either common pericytic marker α -smooth muscle actin (monoclonal anti- α SMA clone 1A4, 1:200, Sigma), vascular smooth muscle cell marker smooth muscle myosin heavy chain (anti-SMMHC, 1:800, Sigma), contractile smooth muscle cell marker calponin (anti-calponin, 1:800, Sigma), pericytic and smooth muscle cell progenitor marker platelet-derived growth factor receptor- β (anti-PDGFR β 1:800) or basement membrane marker collagen IV (anti-COLIV, 1:500, Sigma) was combined with anti-vWf. Cells were washed three times with PBS, fixed with ice-cold 70% ethanol for 20 min, permeabilized with 0.5% Triton X-100 (JT Baker, Phillipsburg, N.J., USA) for 15 min and blocked for unspecific staining with 10% bovine serum albumin (Sigma) for 30 min. After blocking, cells were incubated with the primary antibody pairs for 1 h at room temperature. Cells were washed three times with PBS, incubated for 30 min with secondary antibody polyclonal anti-rabbit IgG TRITC (1:100, Sigma) for anti-vWf and polyclonal anti-mouse IgG FITC (1:100, Sigma) for anti- α SMA, anti-COLIV, anti-PDGFR β and anti-SMMHC. Cell nuclei were stained with Hoechst 33258 (1 μ g/ml, Sigma) for 5 min and washed 5 times with PBS. For anti-GFP staining, the primary antibody pair was mouse monoclonal antibody to GFP (1:100, Abcam, Cambridge, UK) and anti-vWf, secondary antibodies being anti-mouse IgG TRITC (1:100, Sigma) and polyclonal antibody to rabbit IgG FITC (1:500, Acris Antibodies GmbH, Hiddenhausen, Germany), respectively. Fluorescence was visualized with a Nikon Eclipse Ti-S microscope (Nikon, Tokyo, Japan) or with a confocal laser scanning microscope Zeiss LSM 700 (Zeiss LSM 700 on the Axio Observer, Carl Zeiss Microimaging GmbH, Jena, Germany) and the images were processed with Zen2009 (confocal images, Carl Zeiss), with Adobe Photoshop software 7.0 (Adobe Systems, San Jose, Calif., USA) and Corel Draw software 10.0 (Corel Corporation, Ottawa, Ont., Canada).

Microscopic Analysis of Tubule Formation

After immunocytochemical staining, the tubules were analyzed with Nikon Eclipse TS100 microscope (Nikon, Tokyo, Japan) from 48-well plate wells with 40 \times magnification. The extent of tubules in different cultures was quantified visually by using a semiquantitative grading scale from 0 to 10. The grading was based on tubule formation, the length and the branches of tubules, as described in our previous study [Sarkanen et al., 2011].

Statistical Analysis

Statistical analyses were performed and graphs processed with GraphPadPrism 5.0 (GraphPad Software Inc., San Diego, Calif., USA). Tubule formation and RT-PCR results were subjected to one-way ANOVA followed by Dunnett's and Bonferroni's post-hoc tests when applicable. The results were reported as mean \pm SD and differences were considered significant with $p < 0.05$, $p < 0.01$ and $p < 0.001$.

Results

The Tubule Network Formation Is Induced in hASC Monoculture and in hASC+HUVEC Coculture in the Presence of Inductive Growth Factors

The tubule formation capacity and anti-vWf-positive endothelial tubule structures of hASC monoculture or hASC+HUVEC coculture were evaluated and compared at two different time points (day 3 and day 6) when grown in the growth factor-enriched EGM-2 BulletKit medium (fig. 1a). hASC+HUVEC coculture showed an extremely accelerated proliferation rate that was shown at day 6 as a massive, dense, multilayered prevascular-like network formation. hASC+HUVEC coculture tubular network formation was very extensive early (day 3) in vitro, always reproducible and not dependent on the cell line or passage number of cells. In hASC monoculture, the induction towards angiogenesis was not seen repeatedly between different experiments, only approximately half of the cultures showed tubule formation in some degree and dense tubular network formation, as seen in figure 1a, was seen less often. When the tubule formation was semiquantitatively evaluated (fig. 1b, tubule formation), hASC+HUVEC had significantly more tubules at day 6 than at day 3 ($p < 0.001$) and significantly more tubules than hASC monoculture at day 3 ($p < 0.01$) and at day 6 ($p < 0.01$). The two controls, hASC+HUVEC grown without growth factors and HUVEC alone, both grown in growth factor-enriched EGM-2 medium, showed only mild tubule formation or no tubule formation, respectively (fig. 1c). The surface marker characterization of control HUVEC is shown in table 2. We also studied the hASC+HUVEC tubule formation in EGM-2 when FBS was replaced with 2% human serum (hASC+HUVEC EGM-2, 2% HS, fig. 1d) or when FBS was completely removed (hASC+HUVEC, without serum, fig. 1d). We were able to replace FBS without significant effect on the tubule formation capacity of hASC+HUVEC coculture as tested with one-way ANOVA.

In order to further evaluate which cells were responsible for the massive tubule network formation in hASC+HUVEC coculture and to see whether hASC induce the proliferation and tubule formation of HUVEC or also differentiate into endothelial-like cells, as seemed to be the case according to hASC monoculture studies, we created a hASC and GFP-infected HUVEC coculture (hASC+GFP-HUVEC, fig. 2). The anti-vWf-stained cocultures (fig. 2, vWf) were mostly mosaic and consisted of two different populations of vWf-positive cells, approximately half of the cells being GFP infected (fig. 2,

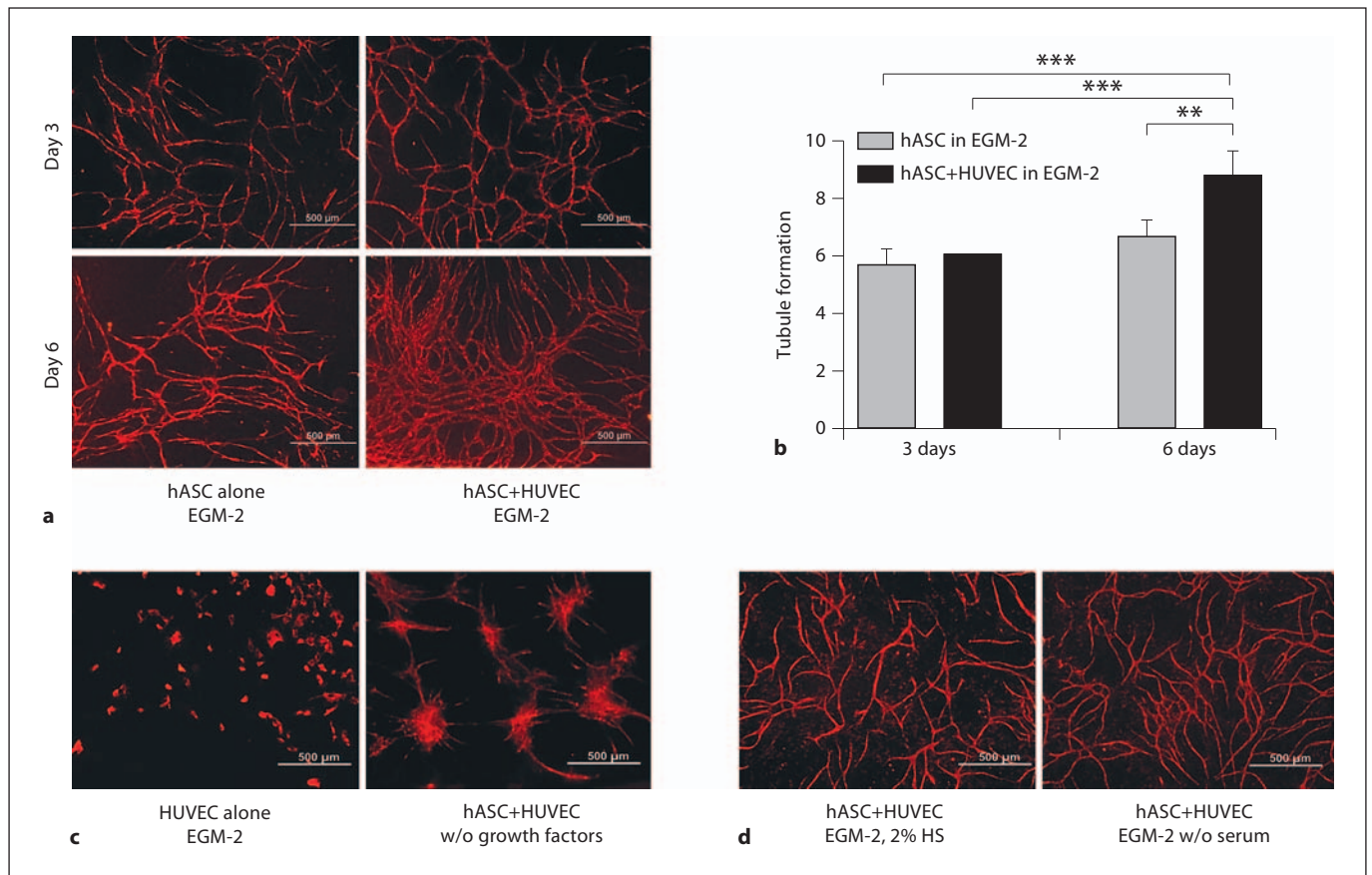


Fig. 1. The tubule formation of hASC monoculture and hASC+HUVEC coculture. Cells were stained with vWf antibody (anti-vWf, 1:500, Sigma; red fluorescence shown with TRITC-conjugated secondary antibody, 1:100, Sigma). **a** Comparison of tubule formation of hASC monoculture and hASC+HUVEC coculture. Cells were cultured and induced to angiogenesis for 3 or 6 days in growth factor-enriched EGM-2 BulletKit medium. **b** Semi-quantitative analysis of the tubule formation between different treatments. hASC monoculture and hASC+HUVEC cocul-

ture were compared to each other at 3 and 6 days. Semiquantitative scale according to Sarkanen et al. [2011]. The results are reported as mean \pm SD and differences considered significant when ** $p < 0.01$ and *** $p < 0.001$. **c** The controls shown at day 6 in vitro. HUVEC were plated at 4,000 cells/cm² and grown in growth factor-enriched EGM-2 and hASC+HUVEC cocultured without exogenous addition of growth factors. **d** hASC+HUVEC were cultured in the growth factor-enriched EGM-2 with human serum (EGM-2, 2% HS) or without serum (EGM-2 w/o serum).

Table 2. Surface protein expression of HUVEC

Antigen	Surface protein	HUVEC
CD 13	Aminopeptidase N	99.4
CD 31	PECAM	94.5
CD 34	Glycoprotein	3.9
CD 44	Hyaluronate, HCAM	44.5
CD 63	Tetraspanin	8.5
CD 117	c-kit, SCFR	8.4
CD 133	AC133	1.1
CD 144	VE-Cadherin	0.9
CD 202b	Tie-2/TEK	31.9
	VEGFR2	0.4
	vWF/factor VIII	14.5

GFP), and the other half of the anti-vWf-positive cells being GFP negative. Moreover, several GFP-negative and vWf-negative cells were surrounding the tubules as seen with Hoechst staining (fig. 2, merged).

Genes That Indicate Differentiation of Cells and Maturation of Tubule Structures Are Upregulated in hASC Monoculture and in hASC+HUVEC Coculture

hASC and hASC+HUVEC, both cultured in EGM-2 BulletKit medium, were further tested for their maturation stage with several pericytic and smooth muscle markers. The relative mRNA expression of *PECAM-1*, *Angiopoietin 1* and *Caldesmon* were studied both in

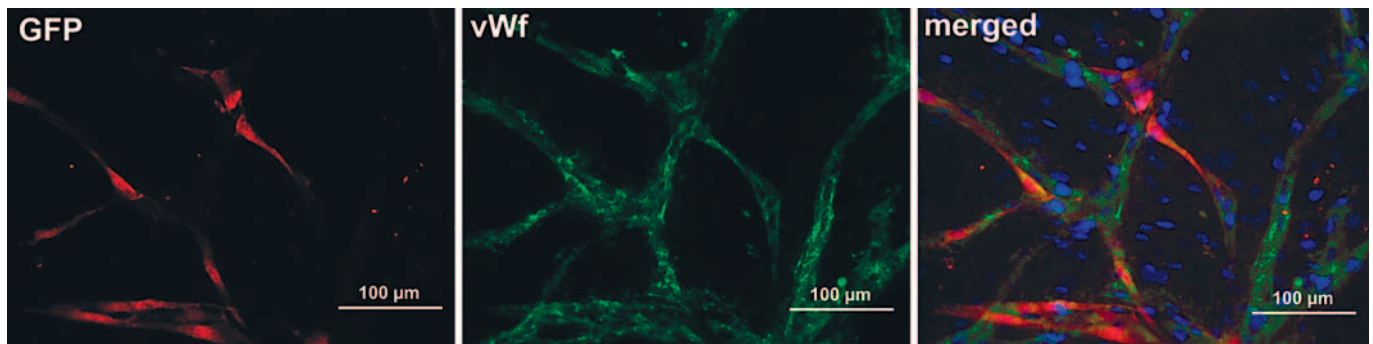


Fig. 2. hASC+GFP-HUVEC coculture model. Cells were cultured for 6 days in the growth factor-enriched EGM-2 medium and immunostained with anti-GFP (GFP, 1:100, Abcam; TRITC-conjugated secondary antibody, 1:100, Sigma) and anti-vWf (1:100, Sigma; FITC-conjugated secondary antibody, 1:500, Acris Antibodies). Cell nuclei were visualized with Hoechst staining (shown in the merged image, 1 μ g/ml, Sigma).

hASC monoculture and in hASC+HUVEC coculture at days 3 and 6 (fig. 3). As a control, hASC, grown in their normal culture media, were used. By day 3, only *Caldesmon* showed significant activity, when compared to the control. *Angiopoietin 1* and *Caldesmon* expressions were significantly increased by day 6 with hASC and with hASC+HUVEC by days 3 and 6 ($p < 0.05$ and $p < 0.001$, respectively) when compared to the control hASC. The *PECAM-1* expression was significantly increased with hASC grown in growth factor-enriched medium by day 6 ($p < 0.05$) when compared to the control hASC, and moreover, also between days 3 and 6 ($p < 0.05$). *PECAM-1* expression was also seen to increase with hASC+HUVEC, but due to high variation, the result was not statistically significant. *Caldesmon* expression was increased between days 3 and 6 with hASC and with hASC+HUVEC ($p < 0.01$ and $p < 0.001$, respectively). It seemed that the *PECAM-1* expression was lower with hASC+HUVEC than with the control hASC monoculture. However, the *PECAM-1* expression was very low at day 3 in all treatments, which has probably caused an error in the normalization of the data.

Induced Extracellular Matrix Production in Culture and Differentiation of hASC into Vessel Lining Cells

hASC monoculture and hASC+HUVEC coculture were also subjected to immunocytochemical staining. The results from day 6 are shown in figure 4, except for PDGFR β expression in hASC+HUVEC which is shown from day 3. PDGFR β expression was most intense in hASC+HUVEC at day 3, seen as dot-like structures evenly surrounding the developing tubules. At day 6, PDGFR β was seen in some extent in both hASC and

hASC+HUVEC, but the expression was not changed with hASC+HUVEC between days. COLIV, showing the development of a basement membrane, was remarkably widely expressed in hASC+HUVEC. The expression was colocalized with the developing tubules covering the tubules. α SMA- and SMMHC-positive cells were expressed quite often in hASC and widely in hASC+HUVEC at day 6, often localized in the branching points of tubular structures and between the tubules. SMMHC expression was increased between days 3 and 6, especially in hASC+HUVEC. In hASC monoculture, vessel-supporting pericytic and smooth muscle cell markers were expressed, even despite the lack of capillary formation at the area. COLIV was also expressed in hASC monoculture at the sites of tubule formation, but not elsewhere. The confocal laser scanning microscopy of basement membrane (COLIV) and tubule network (vWf) staining showed the multilayered nature of the tubule network (fig. 5). The transparent three-dimensional projection of the Z-stack shown in figure 5a was merged from 28 single layers, the distance between layers being 7 μ m. Therefore, the thickness of the tubule network was 196 μ m. The close-up of the tubule network (fig. 5b) shows multilayered tubules crossing each other. Some of the crossing points are indicated with arrows.

Discussion

We developed an inductive prevascular-like network support based on adipose stromal cells and endothelial cells for in vitro tissue models and for tissue engineering constructs. Our specific aim was to develop a completely

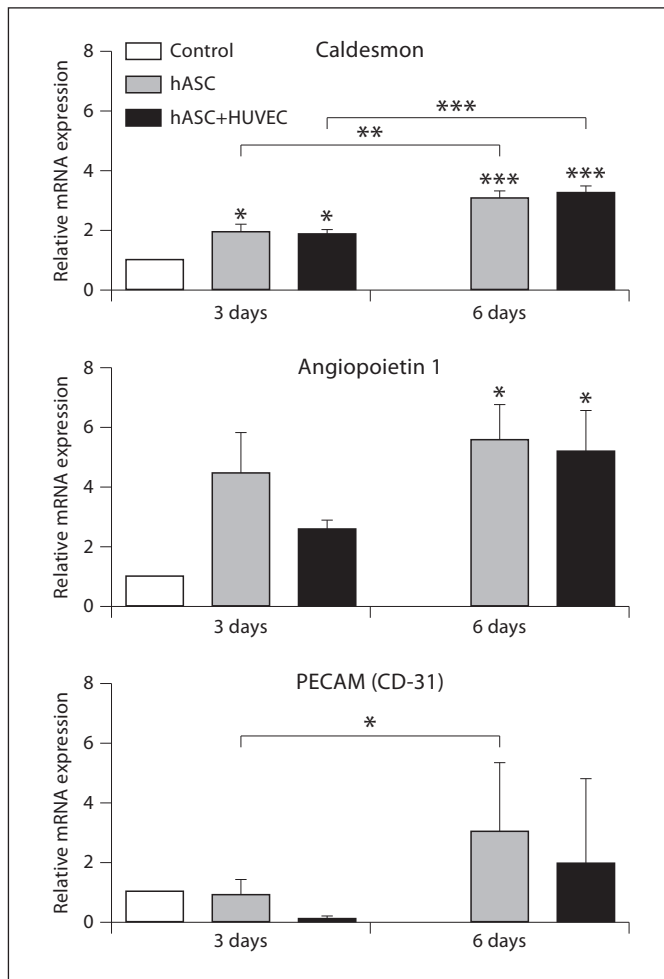


Fig. 3. The relative mRNA expression of *PECAM-1*, *Caldesmon* and *Angiopoietin 1* in hASC monoculture or hASC-HUVEC coculture at 3 and 6 days after angiogenesis induction with growth factor-enriched EGM-2 medium. The expressions were compared to their respective control (hASC, plated at 20,000 cells/cm², grown in hASC culture medium). The statistical analysis was performed with one-way ANOVA. The results are reported as mean \pm SD and differences considered significant when * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

human-based, simplified and improved angiogenesis model, a tubular network with properties of maturing vessels, which could be used for studying angiogenesis in vitro, and that would aid in the development of tissue models by improving target cell proliferation, survival and differentiation. In this tubular network model cells are induced with natural growth factors and allowed to self-assemble into a prevascular-like network and vascular supporting structures. We studied a hASC+HUVEC coculture model by combining some properties and anal-

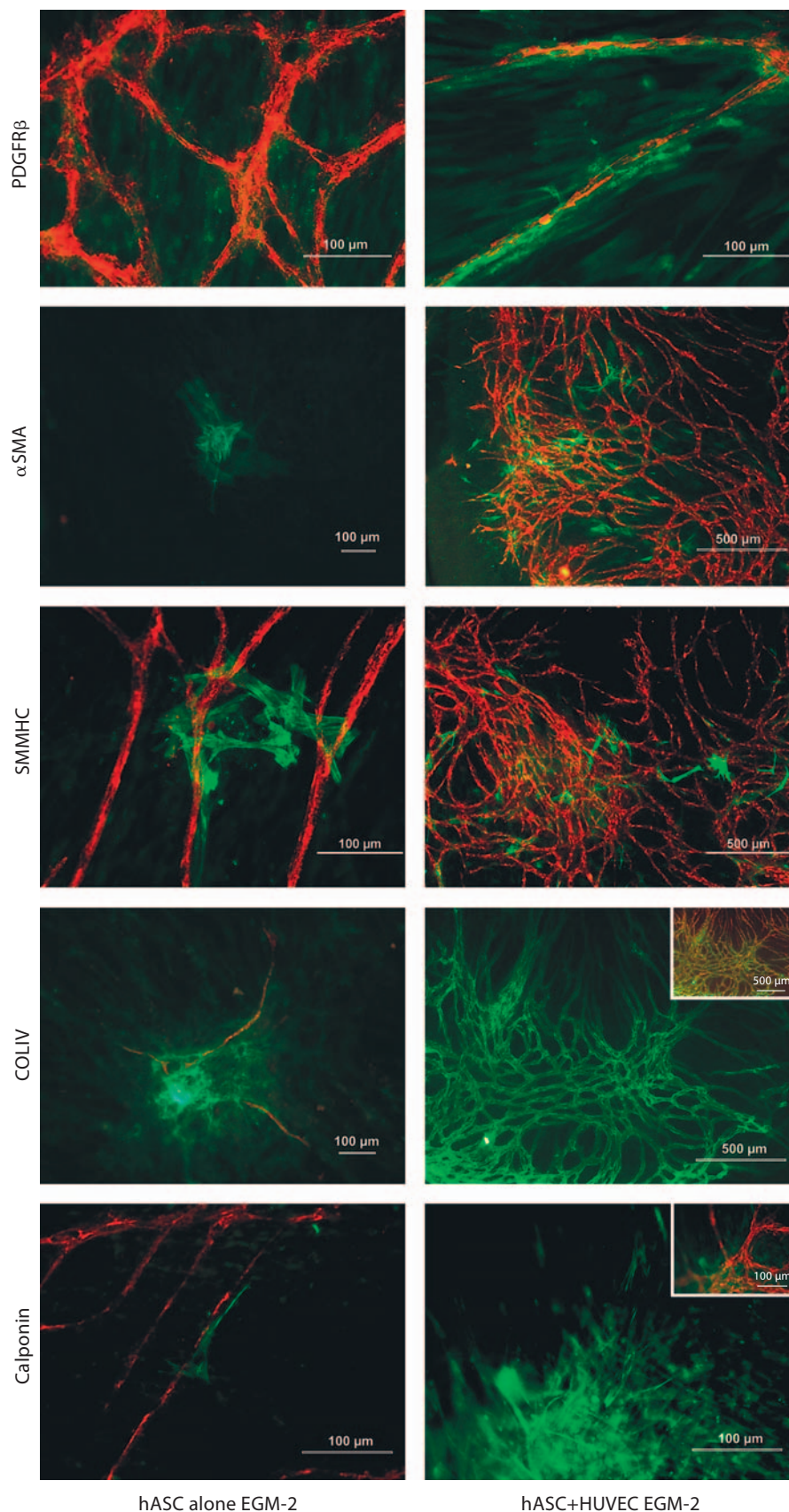
yses from the previously described model [Merfeld-Clauss et al., 2010] and the fibroblast-HUVEC coculture angiogenesis assay previously validated in our laboratory [Sarkanen et al., 2011]. The maturity of the developed model was evaluated with markers that indicated the maturation of the tubules by quantitative RT-PCR and immunocytochemical staining. We also studied whether hASC alone could be induced to endothelial cell differentiation and tubule formation with the addition of these naturally occurring stimulating growth factors.

Merfeld-Clauss et al. [2010] created a similar hASC+HUVEC coculture model with no exogenous growth factors added in cell culture. Contrary to their study, we specifically wanted to investigate tubule formation in a similar coculture system by adding growth factors that are secreted in vivo and that naturally stimulate angiogenesis, e.g. in adipose tissue [Hausman and Richardson, 2004; Mehta and Besner, 2007]. This selection of growth factors would provide a close to natural-like cell culture environment and presumably therefore favor the tubule network induction and allow sustained vessel formation and long-term stability of the tubular network, a feature that is often required in the development of complex tissue or organ models.

When hASC were combined with HUVEC (hASC+HUVEC) and cultured in EGM-2, a remarkable enhancement in cell proliferation, capillary formation and induction of maturation was seen with both cells. However, if the hASC were allowed to attach and proliferate in culture for a prolonged time period prior to HUVEC seeding, only modest tubule formation was seen (data not shown). Therefore, only simultaneous plating of cells effectively induced the self-assembly of the multilayered tubule network. The growth factor stimulation induced tubule formation that seemed not to be passage dependent (up to passage 7 tested) with either HUVEC or hASC, a result contrary to previous studies on these cell types cultured alone by us [Sarkanen et al., 2011] and others [Heydarkhan-Hagvall et al., 2008] that have shown that the differentiation capability is passage dependent.

hASC monoculture, on its behalf, had clearly passage and donor-dependent differentiation capacity towards endothelial-like cells despite the use of the growth factor-enriched medium. hASC alone often formed short cords in culture, but only occasionally massive tubule network formation. In addition to the current study, the tubule formation in hASC monoculture has only been shown previously by Miranville et al. [2004] and Heydarkhan-Hagvall et al. [2008]. Several groups have reported cord formation of hASC in Matrigel for example, but it has also been re-

Fig. 4. The expression of pericytic and smooth muscle cell differentiation markers in tubule structures after angiogenic induction with growth factor-enriched EGM-2 medium. For detection of tubule formation, cell cultures were immunostained with vWf antibody (anti-vWf, 1:500, Sigma; red fluorescence shown with TRITC-conjugated secondary antibody, 1:100, Sigma). For detection of tubule maturation, cultures were immunostained with either anti- α SMA (1:200, Sigma), anti-COLIV (1:500, Sigma), anti-PDGFR β (1:500, Sigma), anti-SMMHC (1:800, Sigma) or anti-calponin (1:800, Sigma), all of these green fluorescence, FITC-conjugated secondary antibody (1:100, Sigma). The images shown are merged images of double immunofluorescence at day 6, except for anti-PDGFR β , which is at day 3, and except for anti-COLIV and anti-calponin for which both the merged image of staining (**insets**) and the FITC-conjugated secondary antibody – anti-COLIV/anti-calponin staining – are shown.



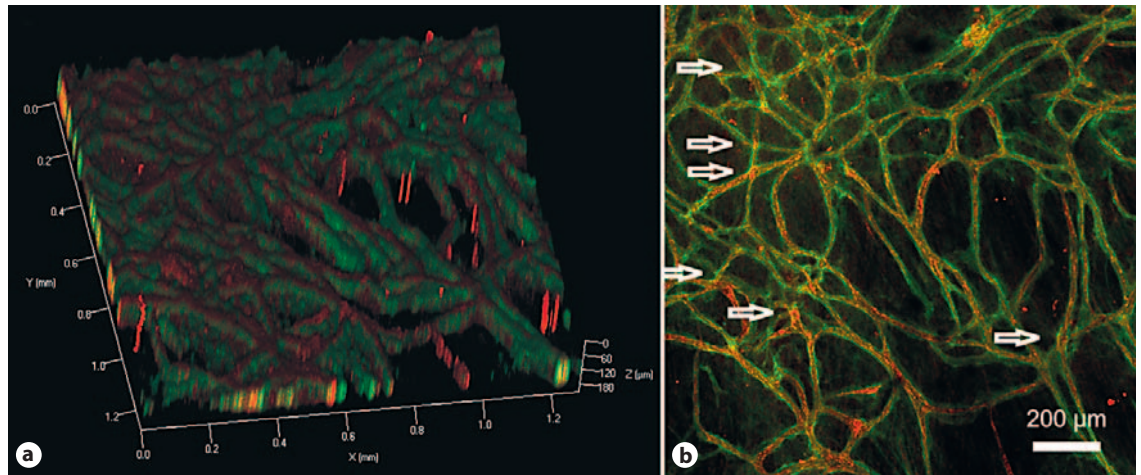


Fig. 5. The confocal laser scanning microscopy of a multilayered precapillary-like tubule network. For detection of the tubule formation, the hASC+HUVEC cocultures were immunostained with vWf antibody (anti-vWf, 1:500, red fluorescence, shown with TRITC-conjugated secondary antibody, 1:100, Sigma). For detection of basement membrane, cultures were immunostained with anti-COLIV (1:500, Sigma green fluorescence, FITC-conjugated

secondary antibody, 1:100, Sigma). The images shown are merged images of double immunofluorescence at day 6. **a** Transparent three-dimensional projection of the Z-stack built from 28 layers with a threshold of 13; distance between layers: 7 μm ; scale bar = 200 μm . **b** A close-up of the tubule network, multilayered tubule formation seen in several areas, some of the crossing points of the tubules are indicated with arrows.

ported that the cord formation observed by different cells in Matrigel is an unspecific phenomenon [Donovan et al., 2001] and, therefore, not a reliable or comparable endothelial tubule formation assay to coculture assays.

To further evaluate which cells contribute to the tubule formation and whether the hASC mainly stimulate endothelial cell proliferation or take part in the endothelial cell differentiation itself, we created a GFP-infection model. In the hASC+GFP-HUVEC coculture, the tubules were seen to consist of a mosaic of GFP-HUVEC and of other cells that were GFP-negative, although vWf-positive. This finding, with our other result that hASC monoculture is able to form tubular networks, suggests that hASC not only stimulate the tubule formation and maturation, but also differentiate into endothelial-like cells in this system. Moreover, the significant increase in the expression of the endothelial marker *PECAM-1* in hASC monoculture between days 3 and 6 supports this finding.

Although freshly isolated hASC, or more precisely, stromal-vascular fraction cells, may contain microvascular endothelial cells [Rehman et al., 2004; Wosnitza et al., 2007], we have previously thoroughly characterized hASC [Lindroos et al., 2009, 2010] and shown that hASC, cultured in several different proliferation media, lack the expression of hematopoietic and angiogenic markers such as CD31, CD106 and CD146 (surface marker expres-

sion 0.6 ± 0.7 , 0.7 ± 0.7 and 0.4 ± 0.2 , respectively). However, the extent whereby hASC differentiate into endothelial-like cells in this model, especially in higher passages, and the functional and metabolic competency of these differentiated endothelial-like cells need careful further evaluation and characterization. In our opinion, the advantage of this current coculture model is, in fact, the heterogeneity of the hASC, creating and inducing a natural-like environment for the formation of a self-assembling tubule network.

The HUVEC isolated from umbilical cords and used in the assay were previously carefully quality controlled for their tubule formation capacity with low variation between cell batches [Sarkanen et al., 2011], and the surface marker expression profile of HUVEC that has been characterized here corresponds with previous reports [Fukasawa et al., 2006; Ichikawa et al., 2006; Korbliing et al., 2006; Schmidt et al., 2006]. However, the flow cytometry results indicate low expression of VE-cadherin (0.9%) and relatively low expression of vWf (14.5%). VE-cadherin is reported to be low in HUVEC in normal cell culture, whereas significantly upregulated in HUVEC during tubule network formation [Kiran et al., 2011]. vWf expression, on its behalf, is reported to be heterogeneous in endothelial cells and to show regional variations in mRNA levels [Zanetta et al., 2000]. Expression of vWf is reported to be upregulated by the angiogenic growth fac-

tors bFGF and VEGF [Zanetta et al., 2000], which may explain the difference in our flow cytometry and immunofluorescence results.

Blood vessel formation involves not only endothelial tubule formation, but also the maturation of vessels by basement membrane formation and pericyte and vascular smooth muscle cell recruitment and differentiation [Kalluri, 2003]. Therefore, the maturation stage of hASC-induced vascular structures was evaluated. Our study supports the earlier studies [Merfeld-Clauss et al., 2010; Planat-Benard et al., 2004; Rubina et al., 2009; Traktuev et al., 2008] and demonstrates that hASC are able to contribute to vessel formation maturation in vitro by differentiating into vessel supporting structures with pericytic and smooth muscle cell properties. PDGFR β , an important receptor in pericytic cell recruitment to developing tubules [Nishishita and Lin, 2004], was already extensively seen at 3 days in hASC+HUVEC. In contrast to a previous study [Merfeld-Clauss et al., 2010], we detected that hASC monoculture expressed COLIV colocalized with the tubules expressing vWf. hASC also widely expressed α SMA and occasionally other markers, even when capillaries were not present, which is well in line with the known vessel supporting role of hASC [Versijden et al., 2010]. COLIV, an important basement membrane component defining integrity, stability and functionality of the basement membrane [Bonanno et al., 2000; Poschl et al., 2004], was intensively expressed in hASC+HUVEC, both at days 3 and 6, and surrounded all of the tubules completely, indicating well-formed basement membrane. COLIV staining also revealed the multilayered structures where the tubules were organized into three-dimensional networks (fig. 4 and fig. 5). hASC+HUVEC also expressed widely α SMA and SMMHC, the major contractile proteins in smooth muscle cells [Frid et al., 1992]. *Angiopoietin 1*, a molecule regulating blood vessel stabilization [Nishishita and Lin, 2004] and indicating late stages of tubule maturation and stabilization, as well as *Caldesmon*, a smooth muscle cell contraction regulator indicating higher differentiation of smooth muscle cells [Frid et al., 1992], were significantly expressed in both hASC and hASC+HUVEC at day 6. Another regulator of muscle cell contraction, calponin, was rarely seen in hASC monoculture, but often in hASC+HUVEC at the sites of the tubule formation. However, the maturation stage of the tubule networks needs further study, especially with endothelial nitric oxide synthase, an important regulator of angiogenesis, as well as by testing the VEGF independency of the formed tubule network.

Most tissue-engineered constructs are limited in thickness to 1–2 mm due to inadequate vascularization [Norotte et al., 2009]. However, scaffolds, which are often used to create multilayered tissue constructs, may interfere with the cell-to-cell interactions and the cell assembly [Norotte et al., 2009]. The growth factor induction induced a high proliferation of both cell types in hASC+HUVEC coculture. Therefore, this supporting prevascular-like network model could allow for the creation of multilayered, yet scaffold-free, soft tissue constructs. The three-dimensional nature of hASC+HUVEC coculture could be seen in confocal scanning microscopy three-dimensional projection, where the total thickness of the tubule network was nearly 200 μ m. This network model would be especially suitable for engineering tissue constructs that need an intense tubular network. Moreover, due to a high proliferation rate, the cell number used in our study was only one fourth of the number used by Merfeld-Clauss et al. [2010], for example. A lower cell number provides a higher capacity, e.g. for in vitro drug screening studies. The xeno-free modification of hASC+HUVEC coculture allows the use of this tubular network culture model in tissue engineering applications. As suggested by Merfeld-Clauss et al. [2010], the endothelial cells could possibly be replaced with any tissue-specific endothelial cells (e.g. microvascular endothelial cells in adipose tissue) with no effect on tubule formation capability. Therefore, we can suspect that this developed tubular network platform is easily modified to be suitable in several different tissue model applications where a dense tubular network is needed. However, when this coculture system is aimed at human use in tissue constructs, the origin of endothelial cells must meet the need of the target tissue. Moreover, if hASC monoculture tubule network induction could be improved to be repeatable, this would allow an even more simplified tubular network model. However, the tubule formation of hASC alone in growth factor-enriched medium needs further study and more optimization, e.g. through an increase in cell seeding density.

By combining the present knowledge of potential properties of hASC in angiogenesis induction and features from the presently available tubule network assays, we could generate a natural-like angiogenesis in vitro with an excellent prevascular-like network formation capability, reproducibility and stability, and minimal limitations in technical performance (i.e. passaging and culture conditions). The tubule network had morphological and genetic features of maturing capillaries. This tubular network model mimics especially well the naturally oc-

curing angiogenesis in adipose tissue. The hASC monoculture or hASC+HUVEC coculture model provides an ideal platform for tissue engineering applications, both for developing tissue models in vitro or for the development of implantable tissue structures. Clinically relevant in vitro angiogenesis models are also valuable tools for investigating the in vivo angiogenesis process [Lai et al., 2009; Ucuzian and Greisler, 2007], e.g. for the treatment of cancer, macular degeneration or peripheral and coronary vascular diseases [Nillesen et al., 2007; Ucuzian and Greisler, 2007].

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Intra-laboratory pre-validation of a human cell based *in vitro* angiogenesis assay for testing angiogenesis modulators

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The developed standardized human cell based *in vitro* angiogenesis assay was intra-laboratory pre-validated to verify that the method is reliable and relevant for routine testing of modulators of angiogenesis, e.g., pharmaceuticals and industrial chemicals. This assay is based on the earlier published method but it was improved and shown to be more sensitive and rapid than the previous assay. The performance of the assay was assessed by using six reference chemicals, which are widely used pharmaceuticals that inhibit angiogenesis: acetyl salicylic acid, erlotinib, 2-methoxyestradiol, levamisole, thalidomide, and anti-vascular endothelial growth factor. In the intra-laboratory pre-validation, the sensitivity of the assay (upper and lower limits of detection and linearity of response in tubule formation), batch to batch variation in tubule formation between different Master cell bank batches, and precision as well as the reliability of the assay (reproducibility and repeatability) were tested. The pre-set acceptance criteria for the intra-laboratory pre-validation study were met. The relevance of the assay in man was investigated by comparing the effects of reference chemicals and their concentrations to the published human data. The comparison showed a good concordance, which indicates that this human cell based angiogenesis model predicts well the effects in man and has the potential to be used to supplement and/or replace of animal tests.

Keywords: angiogenesis, intra-laboratory method pre-validation, FGF-2, VEGF, tubule formation, *in vitro* assay

INTRODUCTION

Angiogenesis, the formation of new blood vessels, is a multistep process regulated by an interplay of pro- and anti-angiogenic factors. The steps involved are: endothelial cell proliferation, migration, differentiation, and tubule formation, as well as stabilization of newly formed blood vessels by a layer of pericytes and smooth muscle cells (Beilmann et al., 2004; Folkman, 2006; Nillesen et al., 2007). Angiogenesis is involved in critical physiological processes such as in embryonic development, wound healing, and female reproductive cycle (Friis et al., 2003; Berthod et al., 2006; Norrby, 2006), as well as in pathologic processes such as in tumor development and macular degeneration (Friis et al., 2003), rheumatoid arthritis (Friis et al., 2003; Middleton et al., 2004), ischemic diseases (van Weel et al., 2008; Cao, 2009), endometriosis (Rogers et al., 2009), and psoriasis (Folkman, 2006).

Due to complex interactions during angiogenesis, the evaluation of factors that affect angiogenesis would optimally be studied *in vivo* (Auerbach et al., 2003). The most commonly used *in vivo* angiogenesis assays include chick chorioallantoic membrane (CAM) assay, Matrigel plug assay, zebrafish embryo system, corneal micro-pocket assay, rat/mouse hind limb ischemia model, rat aortic ring assay, intradermal angiogenesis assays, and *Xenopus* tadpole assay (Auerbach et al., 2003). These *in vivo* assays are used to measure new blood vessel formation by pro- and anti-angiogenic factors and compounds (Akhtar et al., 2002; Auerbach et al., 2003; Norrby, 2006; Ishikane et al., 2008; Ziche and Morbidelli, 2009), and to study tumor

angiogenesis (Auerbach et al., 2003; Norrby, 2006) or embryonic, and organogenic angiogenesis (Norrby, 2006). Despite the advantage of providing more information on complex cellular and molecular interactions compared to *in vitro* models (Norrby, 2006), animal models are burdened by several disadvantages such as variability, animal-specificity, and unethical procedures (Norrby, 2006; Ishikane et al., 2008). The current *in vivo* assays are not pertinent to human diseases as regards both efficacy and relevance (Norrby, 2006). Therefore, human cell based (*in vitro*) assays would have potential to be more predictive than animal models when investigating the effects in man.

Current *in vitro* angiogenesis assays include cell proliferation and cell migration assays, tubule formation assays, and organ culture assays (Auerbach et al., 2003; Ucuizian and Greisler, 2007). Three-dimensional *in vitro* assays permit cell-to-cell interactions, but responses are often difficult to evaluate and quantitate. Most cell culture assays (e.g., tubule formation assays in collagen or Matrigel, cell motility assays, trans-membrane assays, or cell proliferation assays) only reflect one single step of angiogenesis (Auerbach et al., 2003). An ideal *in vitro* assay would measure both stimulation and inhibition (Bishop et al., 1999; Norrby, 2006) and provide quantitative measurement of the tubule formation (Norrby, 2006). Although tubule formation *in vitro* does not cover the whole angiogenesis process, it effectively mimics the key steps, i.e., migration and differentiation of endothelial cells (Donovan et al., 2001).

The objective of the study was to intra-laboratory pre-validate the optimized test method for testing of angiogenesis modulators in routine use to replace and/or supplement animal experiments. The co-culture assay published by Bishop et al. (1999) and Donovan et al. (2001) was further developed, optimized and finally pre-validated in the laboratory. All critical steps including cell isolation procedure, cell number, cell passage, culture time, and tubule formation procedure as well as microscopic analysis were thoroughly investigated and optimized before the intra-laboratory pre-validation. During the intra-laboratory pre-validation, the performance of the assay was assessed by using six reference chemicals, inhibitors of angiogenesis, with animal and human data available; levamisole hydrochloride (levamisole), acetyl salicylic acid (ASA), thalidomide, erlotinib hydrochloride (erlotinib), anti-vascular endothelial growth factor (anti-VEGF), and 2-methoxyestradiol (2-ME). The relevance of the method to man was shown by comparing the obtained results to the literature data from the clinical studies with the same compounds. We here show that through thorough optimization and intra-laboratory pre-validation, it is possible to develop cell a culture assay into a reproducible and repeatable routine method with minimal variation and easy and fast semi-quantitative analysis of the effects.

MATERIALS AND METHODS

MATERIALS AND CHEMICALS

The BJ human foreskin fibroblasts were purchased from American Type Culture Collection (ATCC, LGC Promochem AB, Borås, Sweden, Cat. No. CRL-2522, Lot No. 57632920). Fetal bovine serum (FBS, Cat. No. 10106), L-glutamine (Glu, Cat. No. 25030), non-essential amino acids (NEAA, Cat. No. 11140), and TrypLE Express (Cat. No. 12604) were obtained from Gibco, Invitrogen, Carlsbad, CA, USA. DAB Substrate Kit (Cat. No. 00-2014) was from Zymed Laboratories Inc, Invitrogen, Carlsbad, CA, USA. EGM-2 bullet kit including SingleQuots-supplements (Cat. No. CC-3162), endothelial basal medium (EBM; Cat. No. CC-3156), EGM-2 SingleQuots-supplements (Cat. No. CC-4176), Gentamicin sulfate 50 mg/ml (Cat. No. 17-518Z), and Amphotericin B 250 µg/ml (Cat. No. 17-836E) were obtained from Lonza Group Ltd., Basel, Switzerland. Recombinant Human Fibroblast Growth Factor-basic 146 AA (FGF-2, Cat. No. 233-FB, Lot. No. HKW3809032, ED₅₀ for inducing proliferation of NR6R-3T3 mouse fibroblasts is typically 0.1–0.6 ng/ml) and Recombinant human vascular endothelial growth factor 165 (VEGF, Cat. No. 293-VE, Lot. No. II2209012, ED₅₀ for inducing proliferation of human umbilical vein endothelial cells (HUVEC) is typically 2.0–6.0 ng/ml) were obtained from R&D Systems, Abingdon, UK. 1,4-Dithiotreitol (DTT, Cat. No. 233156, Molecular Biology Grade) was obtained from Calbiochem, San Diego, CA, USA. Bovine serum albumin fraction V (BSA, Cat. No. 107350940019) was from Roche, Indianapolis, IN, USA. Erlotinib (Lot. No. BS06110030) was a kind gift from Roche Diagnostics GmbH, Mannheim, Germany. Anti-von Willebrand Factor (Anti-vWf) antibody produced in rabbit (Cat. No. F-3520), Neutral Red (NR) Solution (Cat. No. N2889), anti-VEGF (Cat. No. V6627, Lot. No. 088K1260), thalidomide (Cat. No. T144, Lot. No. 097K4601), levamisole (tetramisole hydrochloride, Cat. No. L9756, Lot. No. 088K0753), 2-ME (Cat. No. M6383, Lot. No.

108K4087), and ASA (Cat. No. A5376, Lot. No. 048K0015) were from Sigma Aldrich, Manassas, VA, USA. Biotinylated anti-rabbit IgG (H + L) made in goat (Cat. No. BA-1000) and Vectastain Elite ABC Kit, Standard (Cat. No. PK-6100) were purchased from Vector laboratories Inc, Burlingame, CA, USA. Triton X-100 was from JT Baker, Phillipsburg, NJ, USA. Collagenase I was purchased from Invitrogen, Paisley, Scotland, UK. A 75-cm² filtered cell culture flasks (Nunc EasyFlask™) were from Nunc, Roskilde, Denmark. MycoAlert® Mycoplasma Detection Kit (LT07-118) was purchased from Lonza Group Ltd., Basel, Switzerland.

POSITIVE AND NEGATIVE CONTROLS

The tubule formation potency of each reference chemical was quantified based on the tubule formation potency of the positive control. The negative control compound induced no tubule formation. The positive control was a cocktail of commercial, well known angiogenic factors, VEGF, and fibroblast growth factor 2 (FGFβ or FGF-2), that are widely used to mimic the human tubule formation *in vitro* (Bishop et al., 1999; Cross and Claesson-Welsh, 2001; Donovan et al., 2001; Friis et al., 2003; Ai et al., 2007). The positive control medium consisted of 10 ng/ml VEGF and 1 ng/ml FGF-2 dissolved in endothelial basal medium (EBM). The optimal positive control was chosen by testing different concentrations of VEGF and FGF-2 (Table 1). As the negative control compound, which induced no tubule formation, endothelial basal medium (EBM) without any growth factors was used. Negative control gave the same response as the solvent. Positive control was used in four parallels and negative control in two parallels throughout the procedure in each 48-well plate to ensure the technical validity of the tests. Additionally, the dimethyl sulfoxide (DMSO) control was used in three of the reference chemicals (thalidomide, erlotinib, and 2-ME) as they were dissolved in DMSO. DMSO concentration never exceeded 0.5%.

REFERENCE CHEMICALS

The six reference chemicals and their concentrations used for pre-validation of an *in vitro* angiogenesis assay are shown in Table 2. The rationale behind selection of the reference chemicals were that they are consistent with the defined objectives of the study (known inhibitors of angiogenesis), they represent different types

Table 1 | The growth factor cocktails used in investigation of the optimal positive control, linearity, upper and lower limits and batch to batch variation.

Growth factor cocktail no.	VEGF/FGF-2 concentration (ng/ml)
1	75/75
2	50/5.0
3	25/2.5
4	10/1.0
5	7.5/0.75
6	5.0/0.5
7	2.5/0.25
8	1.0/0.1

Table 2 | Reference chemicals tested in the intra-laboratory pre-validation of an *in vitro* angiogenesis assay.

Chemical name	CAS-RN	Chemical class	Product class	Concentrations tested	Purity	Supplier	Physical and chemical characteristics
Acetyl salicylic acid	50-78-2	Salicylate	Non-steroidal anti-inflammatory drug (NSAID)	10, 100, 500, 1000, 1500, and 2000 μ M	99.9%	Sigma Aldrich	Powder
Erlotinib	183319-69-9	Quinazoline	HER1/EGFR tyrosine kinase inhibitor	0.0005, 0.001, 0.01, 0.1, 1, 10, 25, and 50 μ M	99.9%	Roche Diagnostics	Powder
Levamisole	16595-80-5	Imidazothiazole	Alkaline phosphatase inhibitor	0.01, 0.1, 1, 10, 50, 100, 250, 500, 750, 1000, and 2000 μ M	99%	Sigma Aldrich	Powder
2-Methoxyestradiol	363-07-2	Estradiol metabolite	Tubulin inhibitor	0.01, 0.1, 0.2, 0.4, 0.6, 0.8, 1, and 2 μ M	99.5%	Sigma Aldrich	Powder
Thalidomide	50-35-1	Phthalimide	Immunomodulator, TNF- α inhibitor	10, 100, 200, 300, 400, and 500 μ M	>99%	Sigma Aldrich	Powder
Anti-VEGF	n/a	Human VEGF ₁₆₅ and VEGF ₁₂₁ antibody, IgG fraction of antiserum	Growth factor antibody	0.5, 1, 2.5, 5, 7.5, 10, 25, and 50 μ g/ml	n/a	Sigma Aldrich	Lyophilized powder

of chemicals and reliable and relevant reference data in animals and man is available. No other inducers of angiogenesis than the positive control were included. The concentration range for each chemical was selected prior to the angiogenesis assay. The criteria for the selection of the chemical were non-toxicity, solubility in the test system, and that DMSO concentration not exceeding 0.5% in the test system. Reference chemicals were dissolved according to manufacturer's instructions and further diluted in stimulation medium (=endothelial basal medium supplemented with 10 ng/ml VEGF and 1 ng/ml FGF-2) prior to use.

These reference chemicals are widely studied angiogenesis inhibitors and pharmaceuticals. Anti-VEGF is the first angiogenesis inhibitor that has been approved by the U.S. Food and Drug Administration under trade name Avastin (bevacizumab) for the treatment of colon cancer (Folkman, 2006). According to Folkman (2006) VEGF antibody (trade name Lucentis) and aptamer of VEGF (trade name Macugen) are used for the treatment of macular degeneration; erlotinib (trade name Tarceva), an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor is used for the treatment of lung cancer; thalidomide is used for the treatment of multiple myeloma. ASA has protective effects against colon cancer and cardiovascular disease (Yan et al., 2010). 2-ME is approved by FDA for the treatment of ovarian cancer under trade name Panzem. 2-ME has also been studied in the treatment of rheumatoid arthritis (Brahm et al., 2008), cardiovascular diseases (Dubey and Jackson, 2009), multiple myeloma (Rajkumar et al., 2007), breast cancer (Greenberg and Rugo, 2010), and prostate cancer (Harrison et al., 2010). Levamisole (trade name Ergamisol) has been used to treat worm infestations in both humans and animals and it is tested in clinical trials for the treatment of chronic idiopathic urticaria (Zhang et al., 2009), for colorectal cancer (Quasar Collaborative et al., 2007; Dahl et al., 2009), for malignant melanoma (Verma et al., 2006), and for malaria (Dondorp et al., 2007).

METHODS

Setting up Master cell banks

Quality control (QC) criteria for setting up Master cell banks were as follows: cell cultures were pure with high proliferation capacity and contained no mycoplasma. Cell viability was over 90%. Cell culture was quality-controlled microscopically and always prior to Master cell bank establishment. The cells were tested for mycoplasma contamination with MycoAlert® Mycoplasma Detection Kit according to manufacturer's instructions. The cell lines (human foreskin fibroblasts) were not passaged over 10 times and the passaging of primary cells (HUVEC) was investigated and optimized. No antibiotics were used in cell culture.

Human foreskin fibroblasts. Human foreskin fibroblasts were obtained from ATCC and cultured in Minimum Essential Medium with Earle's salts, w/o L-Glutamine supplemented with 10% FBS, 1% L-glutamine, 1% NEAA, and 1% antibiotic-antimycotic mixture in 75 cm² cell culture flasks in 10 ml of medium at 37°C in 5% CO₂ incubator. The medium was changed and the cells observed microscopically for their morphology and cell proliferation every 3 days. When confluent, the cells were detached with Tryple Express and subcultured in a ratio of 1:9. The cells were cryopreserved in liquid nitrogen, 500 000 cells per vial to create the Master cell bank.

Human umbilical vein endothelial cells. The human umbilical cords were obtained from scheduled cesarean sections with informed consent from Tampere University Hospital (permission No. R08028 from the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland). The isolation of umbilical vein endothelial cells (HUVEC) from human umbilical cord veins was performed as described by Jaffe et al. (1973) but the process was further optimized. The umbilical cord was separated from the placenta and the umbilical vein was cannulated with a 20G needle. The needle was secured by clamping the cord over the needle with

a surgical clamp. The vein was perfused with Umbilical cord buffer solution (UBS; 0.1 M phosphate buffer solution containing 0.14 M NaCl, 0.004 M KCl, and 0.011 M glucose) to wash out blood after which the other end of the umbilical vein was clamped with a surgical clamp. The vein was infused with 0.05% collagenase I. The umbilical cord was incubated in a water bath at 37°C for up to 20 min. After incubation, the collagenase solution containing HUVEC was flushed from the cord by infusing the vein with UBS. The suspension was flushed out into a 50-ml polypropylene tube. The cells were centrifuged at 250×g for 10 min, resuspended in EGM-2 BulletKit medium, seeded into 75 cm² filtered cell culture flasks, and cultured at 37°C in 5% CO₂ incubator.

The isolated HUVEC were daily observed microscopically for their morphology, cell culture purity, and cell proliferation. The medium was changed every 2–3 days. When confluent, the cells were detached with Tryple Express. Pure HUVEC cultures with good proliferation capacity were subcultured at primary culture (p0) in the ratio of 1:2–1:4 and at passages 1 (p1) upward in a ratio of 1:3–1:5. The isolated HUVEC were tested for their tubule formation capacity in the angiogenesis assay up to passage 10. At passage 2 (p2), the cells were cryopreserved in liquid nitrogen, 500 000 cells per vial to create the Master cell bank. The donor-specific batch number was given to each batch stored in the Master cell bank.

Co-culture establishment

BJ fibroblasts were taken from the Master cell bank, thawed, and cultured as above (see Human Foreskin Fibroblasts) for 3 days. On day 3, the BJ fibroblasts were plated into 48-well plates at a cell density of 20 000 cells/cm², and grown for an additional 3 days. On day 3, the HUVEC were taken from the Master cell bank, thawed, and cultured as above (see Human Umbilical Vein Endothelial Cells), separately from BJ fibroblasts, for 3 days. On day 6, the HUVEC cells were carefully seeded on the top of BJ fibroblasts into 48-well plates at a cell density of 4000 cells/cm². The co-culture was then further used for cytotoxicity test and for angiogenesis assay.

Cytotoxicity test

To find out the highest concentration for each reference chemical for the angiogenesis assay, a cytotoxicity test was performed. Both technicians performed individually the cytotoxicity test for each reference chemical. As the cytotoxicity assay, the neutral red uptake (NRU) cell viability assay was used. The co-culture of fibroblasts and HUVEC was first established as described above (see Co-Culture Establishment). Twenty-four hours after co-culture establishment, the test system was treated by exposing with positive or negative controls (see Positive and Negative Controls), or reference chemicals (see Reference Chemicals and Table 1) for 24 h. After exposure for 24 h, the exposure medium was removed and the cells were washed with preheated PBS. Two hundred fifty microliters of NR-medium (25 mg NR/1 ml medium) was added into the wells and incubated for 2 h at 37°C. After incubation, NR-medium was removed and the cells were washed with 250 µl PBS. After that, 100 µl NR-desorption medium (50% EtOH, 1% acetic acid in H₂O) was added into the wells and incubated in a shaker for 20 min, protected from light. After shaking, the cells were allowed to settle down for 5 min. The absorbance was measured at 540 nm with Thermo Scientific Varioskan Flash Spectral Scanning Multimode Reader (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Angiogenesis assay

The angiogenesis assay was performed independently by two technicians and two analysts. The technicians performed the whole assay independently, except that the microscopic analysis was performed independently and blinded by two analysts. For microscopic analysis the samples were coded by a person not directly involved in the study.

The co-culture of fibroblasts and HUVEC was first established as described above (see Co-Culture Establishment). The day after co-culture creation, the co-culture was exposed to the positive or negative controls (see Positive and Negative Controls), the reference chemical treatments (see Table 1) or growth factor cocktails (see Table 2). The cells were cultured at 37°C for 6 days prior to immunocytochemical staining. The media containing either reference chemicals, or growth factor cocktail, or positive control or negative control, were changed once during the assay, on the third day.

Immunocytochemical staining. At day 6 from the onset of the experiment, the tubules were immunostained with anti-vWF. The media were removed and the cells were washed three times with PBS, fixed with ice-cold 70% ethanol for 20 min, permeabilized with 0.5% Triton X-100 (JT Baker, Phillipsburg, NJ, USA) for 15 min and blocked for unspecific staining with 10% BSA (Roche Diagnostics Corporation, Indianapolis, IN, USA) for 30 min. After blocking, the cells were incubated with primary antibody (anti-vWf, 1:5000) at 4°C overnight or for 1–2 h in room temperature (RT). Cells were then washed three times with PBS, incubated for 30 min with the secondary antibody (biotinylated anti-rabbit IgG, H + L made in goat) and washed again three times with PBS. The cells were then incubated with enzyme conjugate solution (Vectastain Elite ABC Kit) for 30 min, after which substrate was added (DAB Substrate Kit). The color development was followed under microscope for 5–10 min and the reaction was stopped with 0.5 M Tris buffer. After staining, 500 µl of Tris buffer was pipetted into each cell culture well and the plates were sealed with parafilm for storage at 4°C until microscopic analysis.

Microscopic analysis. After immunocytochemical staining, the tubules were analyzed with Nikon Eclipse TS100 microscope (Nikon, Tokyo, Japan). The extent of tubules and their branching was quantified using predetermined visually inspected semi-quantitative grading scale from 0 to 8. The analysis and grading required expertise and therefore were performed by scientists. As the microscopic analysis was based on semi-quantitative visual analysis, we especially wanted to test the repeatability of the analysis. Therefore all plates and wells were coded and analyzed individually by two scientists. Figure 1 shows the tubule network formation from negative and positive control.

Statistics. One-way analysis of variance with Dunnett's post test was used for the statistical analysis of the reference chemical results. The linearity of tubule formation was tested with linear regression and precision with coefficient of variation (CV). The day to day variation of technicians was tested with one-way analysis of variance, person to person variation between technicians with unpaired *t*-test and person to person variation between

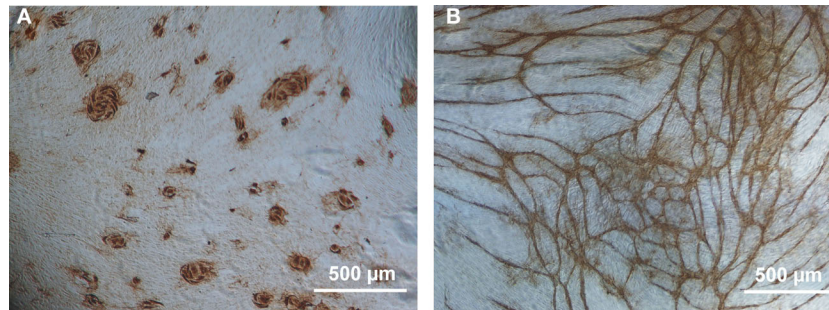


FIGURE 1 | Angiogenesis *in vitro* assay. The BJ fibroblast HUVEC co-culture was immunostained with anti-vWf antibody (1:5000) and with DAB Substrate kit. **(A)** Negative control, no tubule development (value 0 in tubule formation grading). Endothelial cells remain as epithelial-like round areas in co-culture.

(B) Positive control (cocktail of 10 ng/ml VEGF and 1 ng/ml FGF-2) inducing tubule network formation (value 7 in tubule formation grading). Cells form tubule-like structures connecting to each other. Extensive branching of cells and long structures that cover the whole area of the well. Scale bar 500 µm.

analysts with paired *t*-test. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

TEST ACCEPTANCE CRITERIA

Technical criteria

Three wells of the positive control had to give values 6–7 at each testing time. One well could give value 5. Then, the calculated minimum value for positive control is 5.75. The negative control had to be always negative (0). Only one out of two parallels in one combination of each reference chemical during each testing time could be discarded based on visual inspection in case the cells were found to be dead or detached.

Sensitivity of detection

For sensitivity of detection, linearity as well as upper and lower limits of detection, were investigated. Eight different growth factor cocktail concentrations (VEGF and FGF-2, 10:1) were used (Table 1) and investigated in order to find the optimal cocktail for positive control.

Linearity. Linearity was tested in order to find out the growth factor cocktail concentration range that induces tubule formation in a linear manner. For linearity testing, the negative control, and different growth factor cocktails (VEGF and FGF-2, 10:1) were used (see Table 1). Each cocktail was tested in six parallels.

Upper and lower limits of detection. The dose–response effect of the growth factor cocktails were investigated, and the combination of growth factors (see Table 1 for the growth factor cocktails used) giving the maximum value in the linear part of the response curve was selected to be the upper limit of detection. This was also chosen as the fixed positive control for the test (see Positive and Negative Controls). For the lower limit of detection, the growth factor cocktail that gives value 1 in the linear range of the tubule formation scale was selected. Each cocktail was tested in six parallels.

Batch to batch variation

Testing of the batch to batch variation between different HUVEC batches (different umbilical cords, i.e., donor samples) was performed to confirm that each of the Master cell bank batches set

up in our laboratory gives comparable results. The batch to batch variation testing was performed in the angiogenesis assay with the growth factor cocktails shown in Table 1. Three HUVEC batches of passage 4 with 6 parallels were investigated. All of the batches had to follow the criteria for upper and lower limits of detection and to follow linearity.

Precision

To find out the maximal variation due to technicians and microscopic analysts, two technicians performed the positive and negative control tests (each technician performed one 48-well plate of negative control and one 48-well plate of positive control). The negative control was tested in the angiogenesis assay with endothelial basal medium (EBM) without any growth factors. The positive control was tested in the angiogenesis assay with one VEGF and FGF-2 dilution, i.e., positive control (10 ng/ml VEGF and 1 ng/ml FGF-2). Total variation in the test had to be ≤15% analyzed with CV. It is known, that cell culture conditions may be affected by the position of the well in the plate. Therefore, the whole plate was used to test the maximal variation including technicians, microscopic analysts, and cell culture conditions within plate.

Reliability

To test the reliability, two technicians repeated the test three times in unchanged conditions for all the reference chemicals. Reliability included repeatability, i.e., the positive control and the reference chemicals must give comparable results regardless of the testing time, and reproducibility, i.e., the effect of each reference chemical must be the same regardless of the technician. The positive control included altogether 24 parallels performed at three consecutive days by two technicians and each testing time included four parallels. The positive control contained four parallels and was always placed at the same position in a 48-well plate.

Repeatability. The test was performed by two technicians – microscopic analyst – pairs three times using identical protocol (day to day variation).

Reproducibility. Two technician – microscopic analyst – pairs repeated the same identical test protocol (person to person variation). In addition, each analyst analyzed all plates.

Performance

The overall performance of the assay was tested by repeating the identical protocol with six different reference chemicals three times by two technician – microscopic analyst – pairs.

TEST METHOD DATA QUALITY

All work was performed according to the Good Laboratory Practice Regulations as set forth in OECD [ENV/MC/CHEM (98)17], in accordance with OECD guidelines [OECD Guidance document on the Validation and international Acceptance of New or Updated Test Methods for Hazard Assessment (OECD, 2005, No 34) and CPMP/ICH/281/95] and ECVAM (European Centre for the Validation of Alternative Methods) guidance documents (<http://ecvam.jrc.it/>) and according to the standard operating procedures of FICAM.

RESULTS

PRIMARY HUVEC MASTER CELL BANK OPTIMIZATION

The tubule formation potency of HUVEC (treated with the positive control) was studied up to passage 10. The results of the passage optimization are seen in **Figure 2**. It was seen that up to passage 4

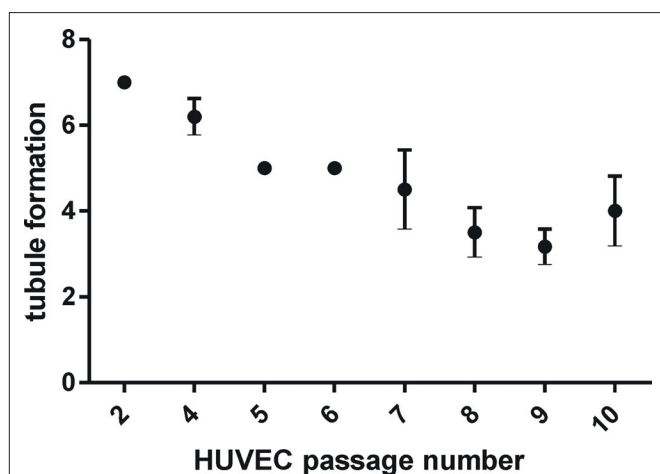


FIGURE 2 | Tubule formation potency of the positive control in the angiogenesis assay at different HUVEC passages. The results are given as mean \pm SD. The results are averages of at least three separate experiments.

the tubule formation remained quite constant and high. During cultivation, when the passage number increased, the tubule formation decreased. Thus, the passage 4 was chosen as the fixed passage to be used in the angiogenesis assay.

CYTOTOXICITY TEST

The concentration giving the viability of 80% or higher was taken as the highest concentration to be used in the angiogenesis assay. Consequently, the concentration ranges were as follows: levamisole 0.01–2000 μ M, thalidomide 10–500 μ M, erlotinib 0.0005–50 μ M, 2-ME 0.01–2 μ M, and anti-VEGF 0.5–50 μ g/ml. Only ASA showed toxicity. The toxicity was found at 2000 μ M. Therefore the concentration range for ASA used in angiogenesis assay was 10–1500 μ M.

ANGIOGENESIS ASSAY

Technical validity of the test

Positive control in each testing time gave the values 6–7 from 3 out of 4 parallels. Negative control always gave 0. No wells needed to be discarded.

Intra-laboratory pre-validation parameters

Sensitivity of detection. The sensitivity of detection was evaluated by studying linearity and the upper and lower limits of detection. **Figure 3A** shows the results obtained with HUVEC in the angiogenesis assay (three batches). The response was linear up to the growth factor cocktail of 10 ng/ml VEGF and 1 ng/ml FGF-2 with all three batches. At cocktails with higher growth factor concentrations, the response decreased. The growth factor cocktail that caused maximal tubule formation in the linear part of the response curve was a combination of 10 ng/ml VEGF and 1 ng/ml FGF-2 (**Figure 3B**) and was the upper limit of detection. This combination was also chosen as the fixed positive control for the test (see Positive and Negative Controls). The lowest VEGF and FGF-2 cocktail combination that induced tubule formation (value 1 in microscopic analysis of tubule formation grading) was found to be a combination of 1 ng/ml VEGF and 0.1 ng/ml FGF-2. This was the lower limit of detection.

Batch to batch variation. The batch to batch variation was tested between three HUVEC batches treated with positive control (each batch with six parallels). The results are shown in **Table 3**. It was

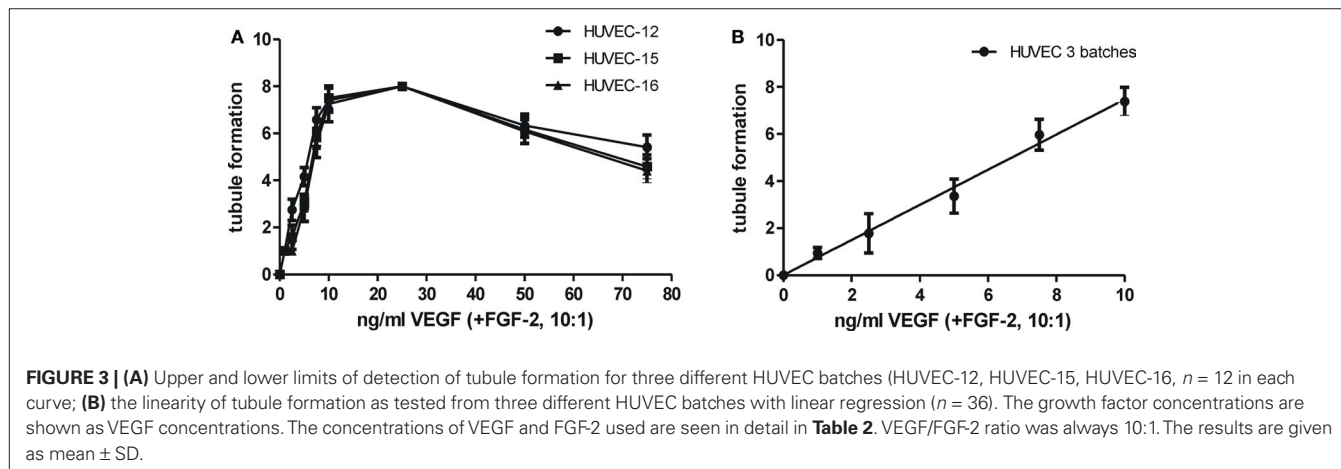


FIGURE 3 | (A) Upper and lower limits of detection of tubule formation for three different HUVEC batches (HUVEC-12, HUVEC-15, HUVEC-16, $n = 12$ in each curve; (B) the linearity of tubule formation as tested from three different HUVEC batches with linear regression ($n = 36$). The growth factor concentrations are shown as VEGF concentrations. The concentrations of VEGF and FGF-2 used are seen in detail in **Table 2**. VEGF/FGF-2 ratio was always 10:1. The results are given as mean \pm SD.

seen, that the criteria for positive control value were met with all batches and that the variation between the batches was extremely small (CV = 1.72%).

Precision. To find out the maximal variation, including that caused by technicians and microscopic analysts, the angiogenesis assay with positive and negative controls was performed. The results of the positive and negative control and their CV% are shown in **Table 4**. Negative control was always found to give the value 0 however; the CV% of positive control was found to be between 6.27 and 7.82%.

Reliability

Repeatability. The results of day to day variation between technicians and microscopic analysts are presented in **Table 5**. No significant differences were observed among technicians ($p = 0.906$ and $p = 0.064$). The microscopic analyst–technician pairs had following day to day variation: microscopic analyst 1 technician 1 CV = 0.59%, microscopic analyst 2 technician 1 CV = 0.67%, microscopic analyst 1 technician 2 CV = 6.38%. Microscopic analyst 2 technician 2 CV = 5.02%. We further studied whether the variation was due to analysis or due to technical performance (see Reproducibility).

Reproducibility. When the person to person variation was studied, each analyst analyzed all plates. Overall there was only a minor difference in CV% between technicians [the results of technician 1 or technician 2 were analyzed by both (two) analysts], being 0.64% with technician 1 and 5.63% with technician 2. The person to person variation is seen in **Table 5**. The results showed that the CV% between microscopic analysts was very low; CV% was 0.34%

Table 3 | Batch to batch variation in tubule formation between three HUVEC batches (HUVEC-12, HUVEC-15, HUVEC-16, $n = 12$ with each batch).

	Criteria	HUVEC-12	HUVEC-15	HUVEC-16
Positive control (mean)	≥ 5.75	7.417	7.500	7.250
Mean (three batches)	≥ 5.75		7.389	
Variation (CV%)	$\leq 15\%$		1.72%	

CV%, coefficient of variation (%).

when the results of technician 1 were analyzed and 2.37%, when the results of technician 2 were analyzed. No statistically significant difference ($p = 0.2084$) was observed between the analysts. When comparing the technicians to each other (person to person variation), a statistically significant difference was observed ($p = 0.007$), whereas no person to person variation between analysts was seen. However, the total CV, i.e., the total variation in the assay was as low as CV = 1.39%.

Performance

The performance of the assay was assessed by six different reference chemicals with several concentrations of each. The results of the effects of reference chemicals on tubule formation are seen in **Figure 4**. All the reference chemicals inhibited tubule formation as expected. Although the response was reference chemical specific, the phenomenon was repeatable, and variation remained constant throughout the study. The tubule inhibition was calculated to be mild (20% inhibition from positive control), moderate (40–60% inhibition from positive control or strong (75–85% inhibition from control). The severity of inhibition and the concentrations causing mild, moderate, or strong inhibition are summarized in **Table 6**, where the reference chemical results of our *in vitro* assay are compared to other *in vitro* angiogenesis test methods, to the relevant plasma concentrations in clinical studies and to effective doses from animal studies.

DISCUSSION

The present intra-laboratory pre-validation study showed that a standardized *in vitro* angiogenesis assay is reliable and reproducible for testing the modulators of angiogenesis, and this human primary cell based *in vitro* assay mimics well the effects in man. Thus, it has great potency to supplement and/or replace animal tests.

Appropriately pre-validated human cell *in vitro* assays are urgently needed for reducing and replacing animal tests. *In vitro* angiogenesis assays are regarded as useful tools for screening compounds and their effective concentrations, but due to complex interactions during angiogenesis, they often need to be followed by *in vivo* studies (Auerbach et al., 2003). Animal assays, although not necessarily predictive for effects in human, are widely used as they provide information on complex cellular and molecular interactions (Norrby, 2006). The disadvantages of the current *in vitro* assays are that they lack metabolism and are rarely completely based on human biology (Auerbach et al., 2003).

Table 4 | The precision of the *in vitro* angiogenesis assay. Maximal variation in the angiogenesis assay was tested with positive and negative controls (Two pairs of microscopic analyst–technician performing each one 48-well plate of negative control and one 48-well plate of positive control).

	Criteria	Technician 1–microscopic analyst 1	Technician 1–microscopic analyst 2	Technician 2–microscopic analyst 1	Technician 2–microscopic analyst 2
Positive control (mean)	≥ 5.75	6.354	6.771	6.896	6.958
Negative control	0	0	0	0	0
Variation (CV%)	≤ 15	7.61	6.27	6.85	7.82

CV%, coefficient of variation (%).

Table 5 | Day to day variation (repeatability) and person to person variation (reproducibility) between the technicians and the microscopic analysts.
Criteria set and the positive control values obtained.

Reproducibility and repeatability					
	Criteria set for positive control/ CV%/p-value	Obtained positive control value			
		Technician 1 –microscopic analyst 1 mean (n = 24)	Technician 1 –microscopic analyst 2 mean (n = 24)	Technician 2 –microscopic analyst 1 mean (n = 24)	Technician 2 –microscopic analyst 2 mean (n = 24)
Day 1 (n = 48)	≥5.75	5.79	5.833	5.75	5.75
Day 2 (n = 48)	≥5.75	5.833	5.833	6.583	6.083
Day 3 (n = 48)	≥5.75	5.875	5.75	6.625	6.5
Mean between days	≥5.75	5.833	5.805	6.319	6.111
Day to day variation among technicians (CV%)	≤15%	0.59%	0.67%	6.38%	5.02%
Day to day variation among technicians (one-way ANOVA)	p < 0.05	ns, p = 0.906		ns, p = 0.064	
Day to day variation between analysts (CV%)	≤15%	0.34%		2.37%	
Person to person variation between technicians		0.64%		5.63%	
Person to person variation between technicians (statistical significance, unpaired t-test)	p < 0.05	p = 0.007			
Person to person variation between analysts (statistical significance, paired t-test)	p < 0.05	ns, p = 0.2084			
Total mean of positive control	≥5.75	6.017			
Total variation of positive control (CV%)	≤15%	1.39%			

CV, coefficient of variation, ns, non-significant.

We further developed, optimized and finally intra-laboratory pre-validated the previously published angiogenesis assay (Bishop et al., 1999). Our assay is based on quality-controlled primary HUVEC that have been minimally expanded *in vitro*. The optimization of the HUVEC passage number was found to be one crucial factor for the adequate performance of the assay. The tubule formation ability decreased significantly after serial passaging. Based on the optimization phase, the use of one, fixed passage of HUVEC was found to be optimal. This shows, that the effect of passage number on the biological activity of cells has to be investigated and the passage number fixed in order to maintain repeatability of the assay. Through optimization, we could obtain a routine assay with minimal variation (overall CV% between the two technicians and microscopic analysts was 1.39%). However, statistically significant difference was found between technicians. The effect of this on the final results is avoided by using positive and negative controls as there was no significant difference in day to day variation among technicians. They are used to calculate the response of unknown substances, as well as used as internal controls in the assay.

The optimal positive control concentration, i.e., VEGF/FGF-2 growth factor cocktail was obtained by investigating the biological response of several growth factor concentrations in several HUVEC batches. The positive control optimization resulted in stable and repeatable effect. The batch to batch variation for HUVEC was minimal due to the optimized passage and pre-set QC criteria for the cells stored in Master cell bank. Moreover, the test duration was shortened from previous methods (Bishop et al., 1999; Donovan et al., 2001) from 14 to 6 days of co-culture and the assay setup was optimized for 48-well plate to minimize the use of cells and to increase the capacity. A semi-quantitative microscopic analysis method, based on tubule formation, their connections and the complexity of the developed network, was developed. The prerequisite for the semi-quantitative grading was that the analysis covered the whole area of each well, contrary to the method of Bishop et al. (1999), where images taken from only five random fields in larger wells (24-well plates) were analyzed by computer analysis program. In optimization phase, the performance assay was tested with 19 reference chemicals;

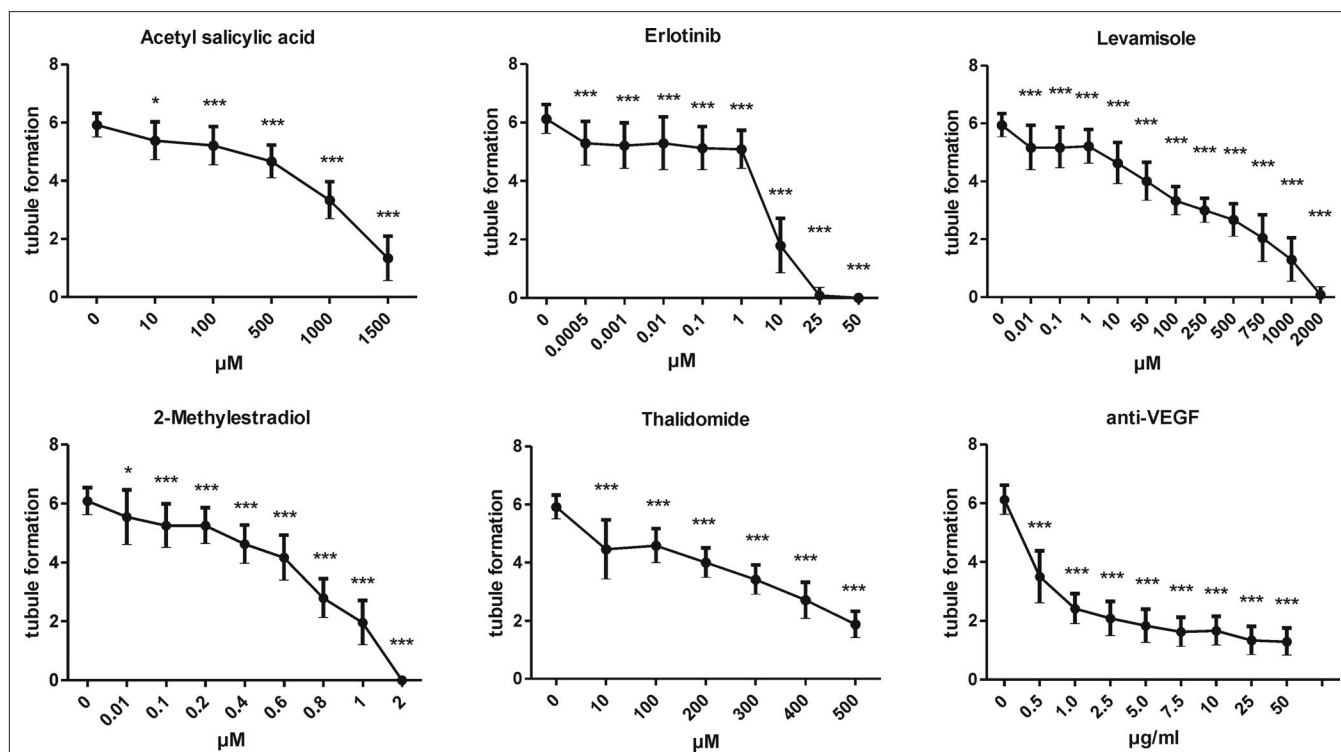


FIGURE 4 | The performance of the assay. The effects of the reference chemicals on tubule formation. The results are given as mean ± SD, $n = 24$ including three testing times with two technicians. Microscopic analysis of all

wells was performed individually by two analysts. The results were tested statistically by using one-way ANOVA with Dunnett's post test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

endostatin, interleukin-1 soluble receptor 1 (IL-1 SR1), suramin, mevinolin, paclitaxel, fumagillin, anti-epidermal growth factor receptor (anti-EGFR) cyclooxygenase-2-specific inhibitor NS-398, indomethacin, VEGF, anti-VEGF, FGF-2, anti-FGF-2, platelet-derived growth factor beta (PDGF-BB), levamisole, erlotinib, ASA, 2-ME, and thalidomide.

The performance of the *in vitro* angiogenesis assay was proven in intra-laboratory pre-validation with six different reference chemicals (levamisole, ASA, thalidomide, erlotinib, anti-VEGF, and 2-ME). The obtained results were compared to the published data from other *in vitro* angiogenesis assays (Table 6). The table shows that comparable results were detected with ASA (Borthwick et al., 2006), with erlotinib (Birle and Hedley, 2006; Jimeno et al., 2007), and with 2-ME (Dobos et al., 2004). However, 2-ME had previously shown low inhibition at 10 μM and moderate inhibition at 50 μM (Kang et al., 2006), whereas our assay showed low and moderate inhibition at 0.01–0.2 and 1 μM, respectively. Moreover, compared to the method by Friis et al. (2003, 2005), which is a similar assay to ours, levamisole was reported to have mild inhibition at 500 μM (in our study 0.01–1 μM), moderate at 750–1000 μM (in our study 100–500 μM), and strong at 2000 μM (in our study 1000–2000 μM). The anti-VEGF was reported to have moderate to strong inhibition at 0.1–10 mg/ml in an *in vitro* model with different study setup (Sims et al., 2008) compared to our assay (inhibition at 0.5–50 μg/ml). There are known to be marked differences in tubule formation depending on the *in vitro* angiogenesis assay

used (Donovan et al., 2001; Auerbach et al., 2003). However, compared to several previous *in vitro* studies, our assay shows improvement of sensitivity.

The relevance of the assay to man was investigated by comparing the obtained reference chemical results with data from clinical studies. The effective concentrations observed in our assay showed very good concordance with the respective therapeutic plasma concentrations (Table 6). The plasma concentrations of anti-VEGF obtained from different clinical trials varied extensively. One explanation is that the endogenous plasma concentrations of VEGF and FGF-2 differ among individuals and are also dependent on the disease (Kakimoto et al., 2002).

In conclusion, we here show that through thorough optimization, it is possible to develop a cell culture assay into a reproducible and repeatable routine method with minimal variation and with easy and fast semi-quantitative quantification of the end points. The intra-laboratory pre-validation study was completed successfully, the test was technically accepted and the pre-set acceptance criteria were met. In comparison of the results to the data from clinical trials shows that this human cell based *in vitro* angiogenesis assay mimics very well the effects in man, and thus it can be used to replace and/or supplement animal tests when testing angiogenesis modulators. The applicability domain contains so far pharmaceuticals. In addition to the chemical testing, the method has potency to test conditioned media of cells or even the effect of different cells (normal or cancer cells) with regard to their effect on angiogenesis.

Table 6 | The comparison of the results from intra-laboratory pre-validated assay to the results from other *in vitro* methods, clinical studies and animal models.

Reference chemical	Results from intra-laboratory pre-validated angiogenesis test method			Results using other <i>in vitro</i> angiogenesis test methods	Results from clinical trials	Results using animal models
	Mild inhibition (% of control) <20%	Moderate inhibition (% of control) 40–60%	Strong inhibition (% of control) 75–85%	Inhibitory effect and concentration	C_{max}	Effective dose (ED)
Acetyl salicylic acid	1.8–8 µg/ml (10–100 µM)	180 µg/ml (1000 µM)	270 µg/ml (1500 µM)	Moderate 500 µM (Borthwick et al., 2006)	<ul style="list-style-type: none"> • 260–1026 µM (Juárez Olguín et al., 2004) • 0–25 µg/ml (Maalouf et al., 2009) • 170 ± 96.7 ng/ml (Bae et al., 2008) 	• 26–300 µM, CAM model (Sharma et al., 2001)
Erlotinib (EGF receptor tyrosine kinase inhibitor)	0.04–40 ng/ml (0.5 nM–0.1 µM)	4 µg/ml (10 µM)	22 µg/ml (50 µM)	Mild 1–20 µM (Birle and Hedley, 2006), Moderate 10 µM (Jimeno et al., 2007)	<ul style="list-style-type: none"> • 0.3–1.13 µg/ml (Herbst et al., 2005) • 0.251–10.7 µg/ml (Ranson et al., 2010a) • 2.93 ± 1.3 µg/ml (Ranson et al., 2010b) • 0.3 µM (Clarke et al., 2010) • 0.56–4 µM (Kraut et al., 2010) 	<ul style="list-style-type: none"> • 50 mg/kg, Mouse carcinoma model (Jimeno et al., 2007) • 50 mg/kg, Mouse tumor model (Cerniglia et al., 2009)
Levamisole	2–240 ng/ml (0.01–1 µM)	25–120 µg/ml (100–500 µM)	240–500 µg/ml (1000–2000 µM)	Mild 500 µM, moderate 750–1000 µM, strong 2000 µM (Friis et al., 2005), strong 2000 µM (Sylvest et al., 2010)	<ul style="list-style-type: none"> • 0.62 µg/ml–1.27 µg/ml (Reid et al., 1998) • 0.716 ± 0.217 µg/ml (Kouassi et al., 1986) 	<ul style="list-style-type: none"> • 1.2–12 mg/kg, Nude mouse tumor model (Friis et al., 2005) • C_{max} 0.37 µg/ml calf parasite infection (Taylor et al., 1988)
2-Methoxyestradiol	3–60 ng/ml (0.01–0.2 µM)	300 ng/ml (1 µM)	600 ng/ml (2 µM)	Mild 10 µM, moderate 50 µM (Kang et al., 2006), Mild 0.5 µM, moderate 1 µM (Dobos et al., 2004)	<ul style="list-style-type: none"> • 3.3 ng/ml (Tevaarwerk et al., 2009) • 30.27 ± 20.18 ng/ml (Matei et al., 2009) • 3.0–21.4 ng/ml (Dahut et al., 2006) • 1.4–13.2 ng/ml (James et al., 2007) • 2.2–9.6 ng/ml (Sweeney et al., 2005) 	<ul style="list-style-type: none"> • 100 mg/kg Murine rheumatoid arthritis model (Plum et al., 2009) • 7.5 mg/kg, 75 mg/kg Mouse tumor model (Dobos et al., 2004)
Thalidomide	2–25 µg/ml (10–100 µM)	77–100 µg/ml (300–400 µM)	-	-	<ul style="list-style-type: none"> • 2 µg/ml (Kakimoto et al., 2002) • 1.68 ± 0.41 µg/ml (Murakami et al., 2009) • 1.44 ± 0.50 µg/ml (Kamikawa et al., 2006) • 0.43–1.03 µg/ml (Vieira and Valente Mdo, 2009) 	<ul style="list-style-type: none"> • 100 mg/kg, Rat Alzheimer model (Ryu and McLarnon, 2008) • 19–1000 µM, CAM model (Sharma et al., 2001)

(Continued)

Table 6 | Continued

Reference chemical	Results from intra-laboratory pre-validated angiogenesis test method			Results using other <i>in vitro</i> angiogenesis test methods	Results from clinical trials	Results using animal models
	Mild inhibition (% of control) <20%	Moderate inhibition (% of control) 40–60%	Strong inhibition (% of control) 75–85%	Inhibitory effect and concentration	C _{max}	Effective dose (ED)
Anti-VEGF	0.01–0.1 µg/ml	0.5–1 µg/ml	25–50 µg/ml	Strong 0.1 mg/ml–10 mg/ml (Sims et al., 2008), Strong 5 µg/ml (Friis et al., 2006), Strong 10 µg/ml (Friis et al., 2003)	<ul style="list-style-type: none"> • 363 µg/ml (Herbst et al., 2005) • 123.2 ± 16.4 µg/ml (Wu et al., 2010) • 11.94–194.08 µg/ml (Ning et al., 2010) • 20.7–24.2 ng/ml (Sharma et al., 2010) • 16.6–42.5 µg/ml (Krohne et al., 2008) • 2.63–165 µg/ml (Zhu et al., 2008) 	<ul style="list-style-type: none"> • 2–4 mg/kg, Mouse carcinoma model (Sims et al., 2008) • C_{max} 104.46 ± 1.44 ng/ml, injection in rabbit (Kim et al., 2010) • C_{max} 1430 ± 186 ng/ml, vitreous injection macaque eyes (Miyake et al., 2010) • C_{max} 676 ± 100 µg/ml, cynomolgus monkeys (Xu et al., 2008)

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*Who's to say
What's impossible
Well they forgot
This world keeps spinning
And with each new day
I can feel a change in everything*

--

*And as my mind begins to spread its wings
There's no stopping curiosity*

--

*Who's to say
I can't do everything
Well I can try
And as I roll along I begin to find
Things aren't always just what they seem*

*I want to turn the whole thing upside down
I'll find the things they say just can't be found*

--

Who's to say what's impossible and can't be found?

from "Upside Down"
Curious George theme
lyrics Jack Johnson